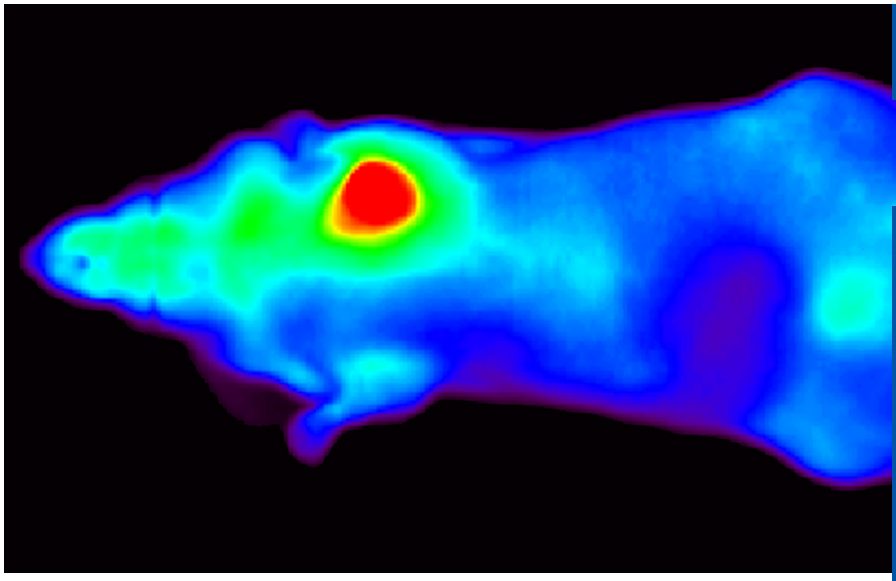

APPLICATION NOTE

Preclinical *In Vivo* Imaging

Authors:

Craig McMannus

Kristine Vasquez

Jeffrey D. Peterson, Ph.D.

PerkinElmer, Inc.
Hopkinton, MA

Vascular Imaging Probes For Oncology and Inflammation Using the IVIS Spectrum

Abstract

Optical-based *in vivo* imaging of vascular changes and vascular leak is an emerging modality for studying altered physiology in a variety of different cancers and inflammatory states. A number of fluorescent imaging probes 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 signal measurement from deep tissue sites. In these studies, we compared three NIR fluorescent probes, Superhance™ 680 (a low molecular weight probe), AngioSense® 680EX (a high molecular weight probe), and AngioSPARK® 680 (30 - 50 nm nanoparticles). Each probe differs significantly in pharmacokinetics, biodistribution, and tissue clearance rates, offering three slightly different imaging tools for cancer and inflammation research. All three probes can detect tumor vascular leak, with AngioSense showing superior signal to background in orthotopic mouse breast cancer. In contrast, Superhance has some distinct advantages in imaging capability when applied to a mouse model of acute, inflammatory paw edema. AngioSPARK, a probe typically used for intravital microscopy assessment of vessel morphometry, can also image both tumors and inflammation with long term accumulation at the target site (sometimes a benefit for time course studies). This research provides guidance regarding optimal usage of these three vascular imaging agents in preclinical mouse cancer and inflammation studies.

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Materials and Methods

Research Animal Use

All experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by PerkinElmer's *(In Vivo* Imaging Division) IACUC committee according to strict guidelines for animal care and use. No invasive or surgical procedures were used in these studies, but all imaging activities were performed under appropriate anesthesia to minimize animal distress.

Fluorescent Agents for the Detection of Cancer and Inflammation

A variety of non-targeted fluorescent agents (Superhance, AngioSense, and AngioSPARK) (PerkinElmer) were used as vascular probes to image blood leakage and accumulation into tumors, and extravasation of probes during edema. The imaging doses were as recommended in the product inserts.

In vivo IVIS® Spectrum Imaging

Mice were anesthetized by isoflurane inhalation. For tumor studies, as nu/nu mice have no hair, depilation was not required. For carrageenan paw edema, depilation was also not required for paw imaging. Mice (n=3 per group) were imaged using the IVIS Spectrum Imaging System (PerkinElmer) which collected 2D epifluorescence images.

IVIS Image Analysis

The collected fluorescence data was analyzed using Living Image® 4.5 system software (PerkinElmer). Two-dimensional regions of interest (ROI) circles were drawn to capture tumor fluorescence, and auto-ROIs were used to precisely outline inflamed and control hind paws. Control ROIs were placed to capture a control site, either a flank region for tumor mice or contralateral paws for CG mice. Additional mice (without paw injection) were used as controls for carrageenan paw edema due to the non-specific vascular leak induced by needle trauma (even with PBS injection). Although background correction is always recommended for accurate interpretation of results, the data is represented in both uncorrected form as well as background corrected for illustration purposes.

Vascular Probe Properties

Fluorescent vascular imaging probes can vary widely, from small molecule dyes to large nanoparticles. The differences in structure, size, and physiochemical properties 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 time points. In order to achieve an effective level of probe in the target tissues (i.e. sites of tumor vascularity, inflammation-based edema, or tumor-associated vascular leak) the probe was imaged *in vivo* immediately upon injection. Vascular dye levels drive the extravasation from circulating blood to the tissue of interest, either accumulating in areas of marked vascular permeability or passively extravagating into normal tissues. Table 1 shows the three different probes examined in the present studies, and Figure 1 shows the blood pharmacokinetics profile.

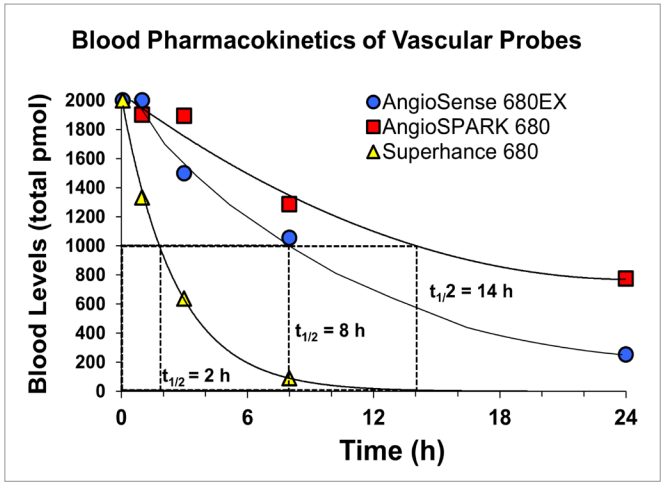

Figure 1. Probes were injected intravenously and blood was collected at the indicated times for assessment by fluorescent microplate assay in comparison to a standard curve.

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

	Superhance 680	AngioSense 680EX	AngioSPARK 680
Probe Type	Albumin-binding Small Molecule	PEGylated Large Scaffold	Nanoparticles
Size	1540 g/mol	70,000 g/mol	30-50 nm
Ex/Em	675/692	670/690	673/690
Blood Half-life	2 h	8 h	14 h
Tissue Half-life	5 h	72 h	> 100 h

Results

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, and others.

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 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 probes offer a useful and safe alternative, particularly for preclinical imaging. Reagents 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 probes were assessed; Superhance 680, AngioSense 680EX, and AngioSPARK 680 each have differences in physical/chemical compositions, blood pharmacokinetics (Figure 1, Table 1), and present slightly different imaging performance *in vivo*. These probes were assessed for their ability to image tumor vascular leak and acute carrageenan paw edema. Probes were assessed by epifluorescence imaging using the IVIS Spectrum system at different time points after injection into mice. In addition, proper analysis and thresholding approaches are highlighted in these datasets.

Tumor Vascular Imaging

We characterized three fluorescent vascular probes in a syngeneic mouse tumor model with orthotopic implantation of 4T1 mouse breast adenocarcima at a single site (in the right upper mammary fat pad) in nu/nu mice. This is a model that has been well characterized with regard to pathogenesis, inflammation, and vascularization/vascular leak, and it has been used to characterize a variety of NIR fluorescent probes, including those detecting cancer-related proteases (cathepsins, matrix metalloproteases, etc.), key receptor biomarkers (bombesin receptor, $\alpha V\beta 3$ integrin, transferrin receptor, folate receptor, etc.), and cell death (annexin). This model, performed as a mammary fat pad implantation, does not require deep tissue imaging for detection, therefore making IVIS Spectrum epifluorescence imaging a robust method for detection of fluorescence changes. As shown in Figure 2, tumors were allowed sufficient time to grow prior to injection of imaging probes, after which imaging datasets were acquired at different time points (tailored to the known pharmacokinetics of each probe) to characterize the optimal kinetics of tumor fluorescence accumulation.

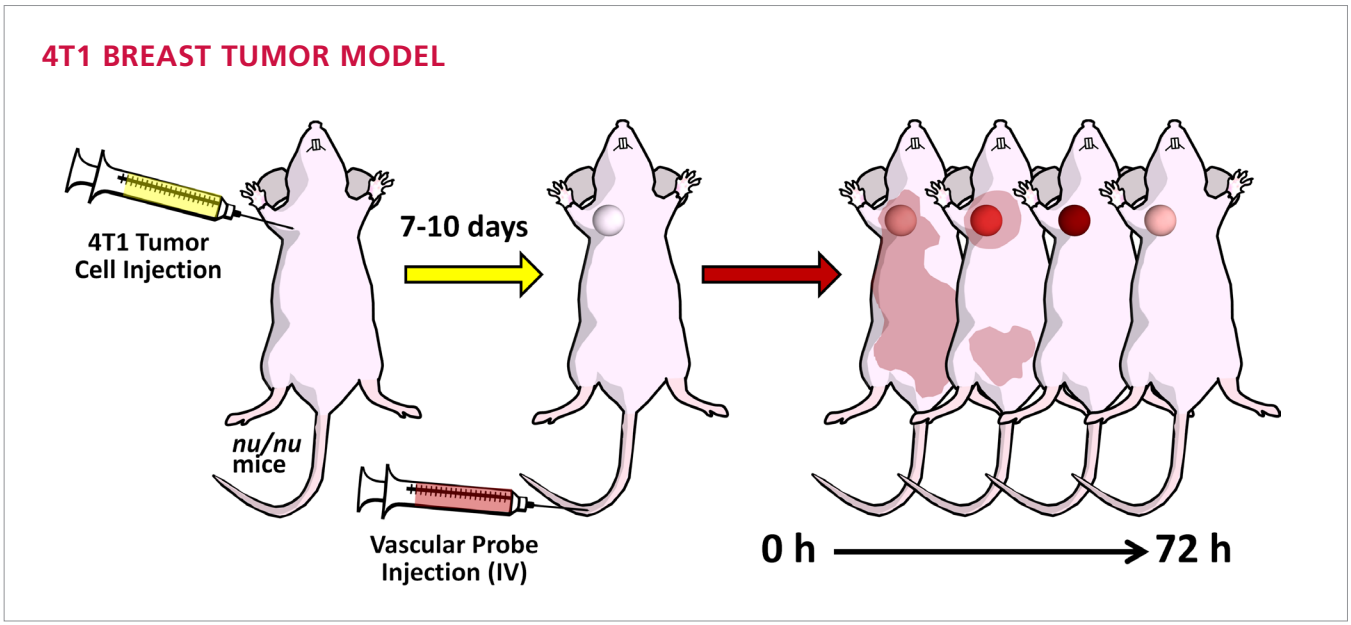

Figure 2. Eight week-old female nu/nu mice were injected in the right upper mammary fat pads with 0.5 x 10^6 4T1 mouse breast adenocarcinoma cells. 7-10 days later, mice were injected with vascular probes and imaged (at indicated times) using the IVIS Spectrum to detect and quantitate levels of fluorescence in tumor sites, control sites, and background regions.

Tumor Vascular Leak Imaging

To determine the optimal imaging time point relative to probe injection, we imaged 4T1 tumor-bearing mice at several different times post-intravenous injection with three of our vascular probes, Superhance 680, AngioSense 680EX, and AngioSPARK 680 at 2 nmols/mouse.

Superhance (Figure 3A), within 30 minutes, showed tumor accumulation, mostly at the tumor margins. Peak tumor signal in whole body images was seen at 3 h post-injection, however significant background signal (predominantly skin) limited the quality of tumor definition. By 24 h, a small decrease in tumor signal, combined with loss of background fluorescence increase the quality of tumor definition. Normalized images (compensating for signal loss) reveal excellent tumor definition even out to 72 h despite 80% signal loss.

AngioSense (Figure 3B) and AngioSPARK (Figure 3C) differ from Superhance in tumor vascular leak imaging due to their larger size and extended blood half-lives. Little or no tumor accumulation is seen until 3 h, with peak tumor accumulation and definition occurring at 24 h. Tumor signal does not decline much through 72 h, providing a long imaging window for these probes.

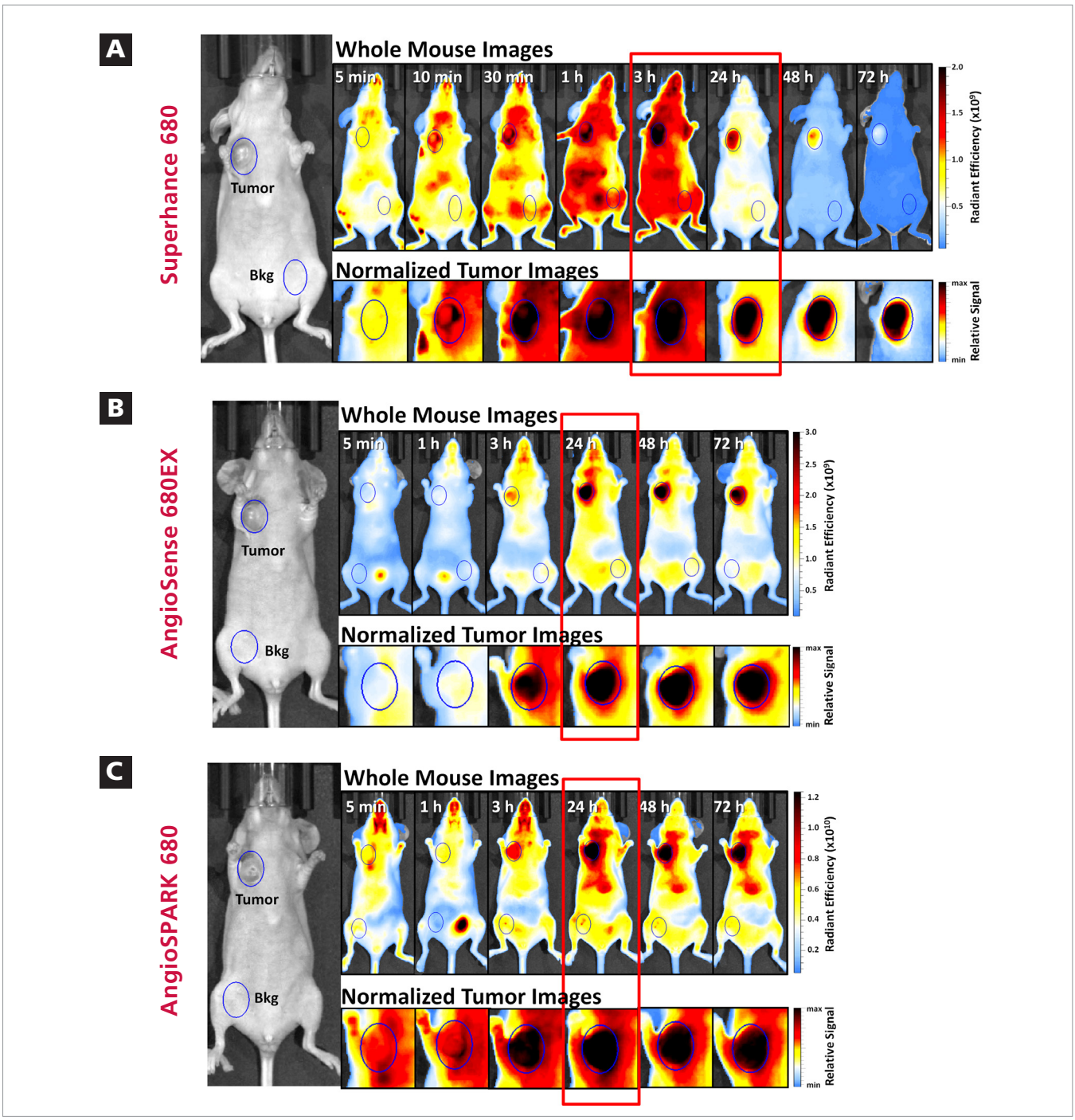

Figure 3. **Vascular probe tumor imaging kinetics.** Representative mice bearing established 4T1 tumors implanted superficially in the mammary fat pad were imaged at different time points following injection of (A) Superhance 680, (B) AngioSense 680EX, and (C) AngioSPARK 680 to show temporal fluorescence localization to tumors. Upper panels, intensity and localization over time; lower panels, normalized images to highlight tumor signal to background. Regions of interest (ROI) for tumor and control signal are indicated.

Quantifying Tumor Vascular Leak

For analysis, tumor and control (flank) regions of interest (ROIs) were placed as shown in Figure 3, and Living Image® 4.5 software and used to quantify signal. Data is shown below in Figure 4 as uncorrected (Figure 4A) and with background correction (Figure 4B) to better illustrate the type of data you will see. Background subtraction can be performed for each individual animal (i.e. tumor – internal flank control), however this zeroes all individual background values, precluding statistical comparison between tumors and background. We instead subtracted the mean background at each time point from individual control and tumor values. This yielded average control values of zero, yet retained individual animal variability and permitted statistical comparison. Background correction is always recommended, as it provides a more accurate quantitative assessment of the data.

Although only three mice per group were used, Superhance 680 tumors achieved statistical significance in comparison to control flank sites from 3-72 h. With regard to selecting optimal times for imaging, one should choose the earliest timepoint(s) at near maximum that also retain statistical significance. For Superhance, this means that 3 h and 24 h are optimal. AngioSense and AngioSPARK both achieve higher tumor signal (due to extended blood PK profiles) and this permits slightly improved statistical significance even prior to achieving peak tumor signal. Both of these probes are recommended for 24 h imaging time points. In particular, AngioSPARK is nanoparticle-based and will accumulate in phagocytic cells within the tumors from 48-96 h post-injection.

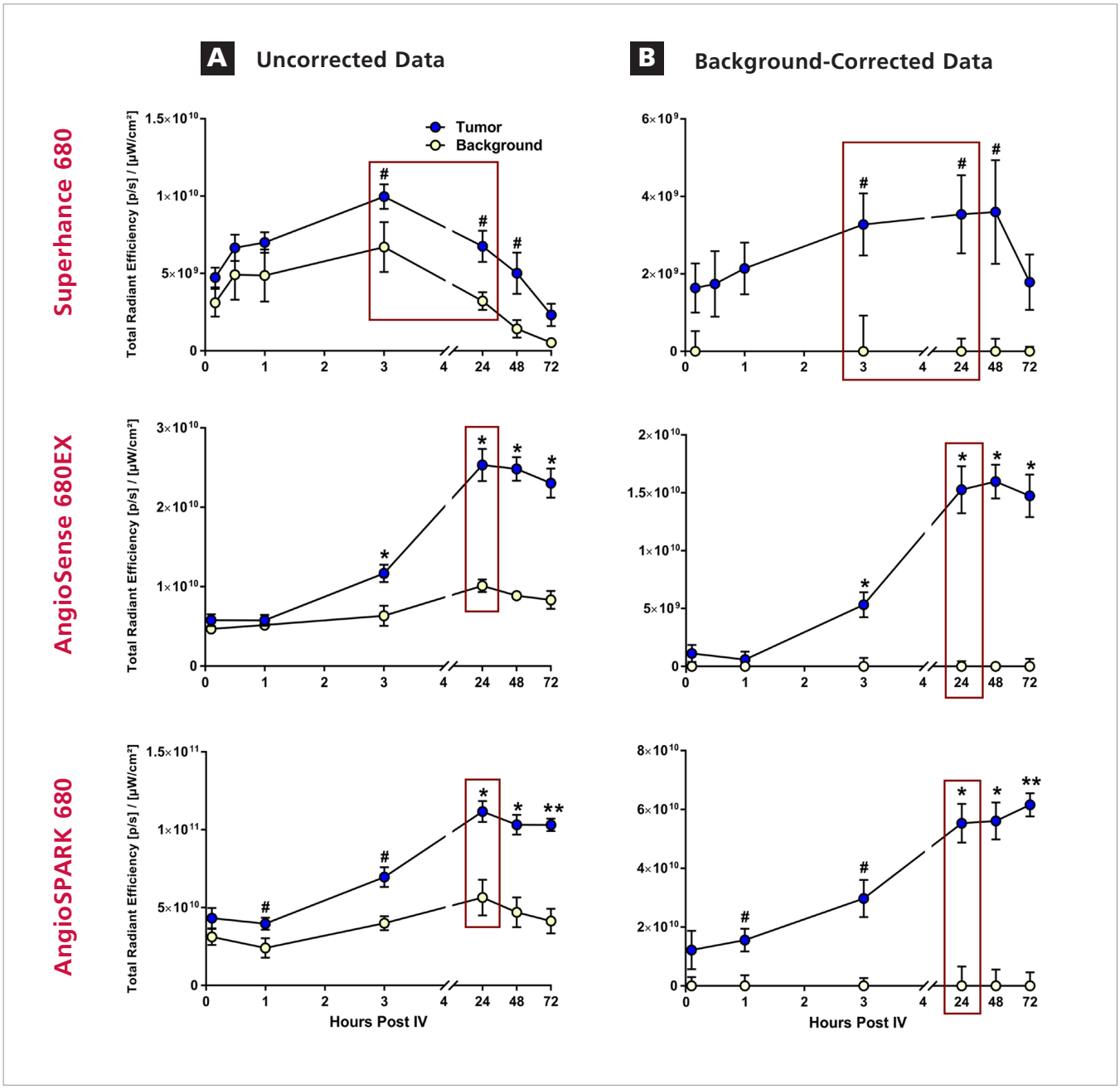

Figure 4. **Quantification and analysis of vascular leak imaging .** (A) Uncorrected and (B) background-corrected data are shown for Superhance 680 (upper), AngioSense 680EX (middle), and AngioSPARK 680 (lower). Statistical differences between tumor and background ROIs were assessed by Student's t test at each individual time point.

Acute Edema Imaging

Carrageenan-induced Paw Edema

Carrageenan-induced paw edema is a widely used inflammation model, used to test new anti-inflammatory drugs as well as to study mechanisms of inflammation. Carrageenan (1% w/v) induces a marked powerful edema in BALB/c mice, with several mediators involved, including COX-2, histamine, serotonin, bradykinin, prostaglandins, and nitric oxide. Following an increase in vascular permeability, neutrophil infiltration produces, among other mediators, oxygen-derived free radicals such as superoxide anion (O2–), hydroxyl radicals, and myeloperoxidase. Neutrophilia peaks at around 3 h post-carrageenan injection, followed by a wave of macrophage infiltration that occurs out to 24-48 h. Paw swelling is the simple, yet common, measure for inflammation severity for this model. Vascular imaging probes, appropriately timed for injection at peak induction of vascular leak, can be used to detect and measure tissue edema (see Figure 5) as an alternative readout to paw swelling (Figure 6). As any paw that is injected with a needle will show some level of vascular leak associated with wound healing, additional controls (un-injected mice with probe injection) can be used to correct for non-specific circulating background signal. This allows quantification of vascular leak associated with needle-stick alone versus the response to carrageenan.

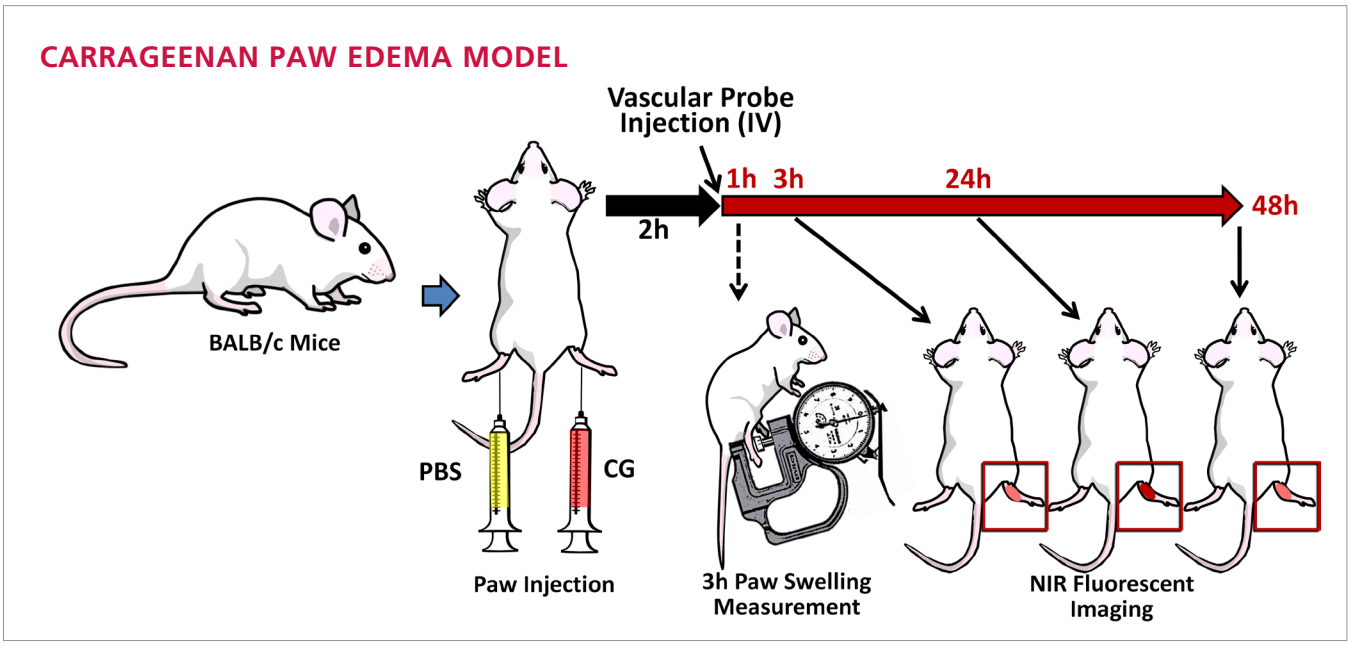

Figure 5. Eight week-old female BALB/c mice were injected in the right footpad 30 ul of 1% Carrageenan (CG), and left footpads were injected with 30 ul PBS to control for the needle-stick-induced background tissue response. Probes were injected 2 h later and mouse paws were imaged 3 h, 24 h, and 48 h post probe injection for quantification of fluorescence in inflamed paws, PBS paws, and uninjected mouse controls.

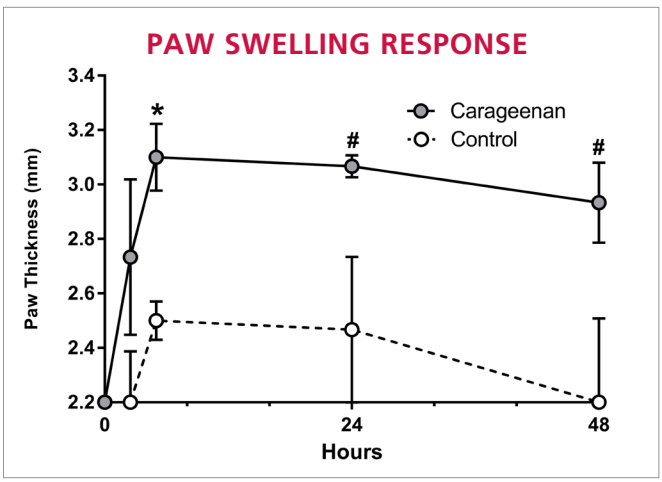

Figure 6. Carrageenan paw edema responses were measured in week-old female BALB/c mice that were injected in the right footpad 30 ul of 1% Carrageenan (CG), and in the left footpad with 30 ul PBS. Paw thickness were measured using a dial thickness gauge (Mitutoyo, Kawasaki Japan) to compare PBS- vs CG-injected paw thickness at different times post-CG.

Acute Edema Imaging

To determine the optimal imaging time points for our three different vascular imaging probes, we injected them IV (2 nmols/mouse) in the plantar surface of the right paw 2 h following intradermal carrageenan injection, a time at which neutrophil influx and edema is on the increase. Left paws were injected with PBS. As shown in Figure 7, all three probes showed increased signal in carrageenan paws, generally peaking at 3-24 h, with differing background signal in control paws. Superhance cleared quickly from tissue, making this probe ideal for imaging early time points in 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.

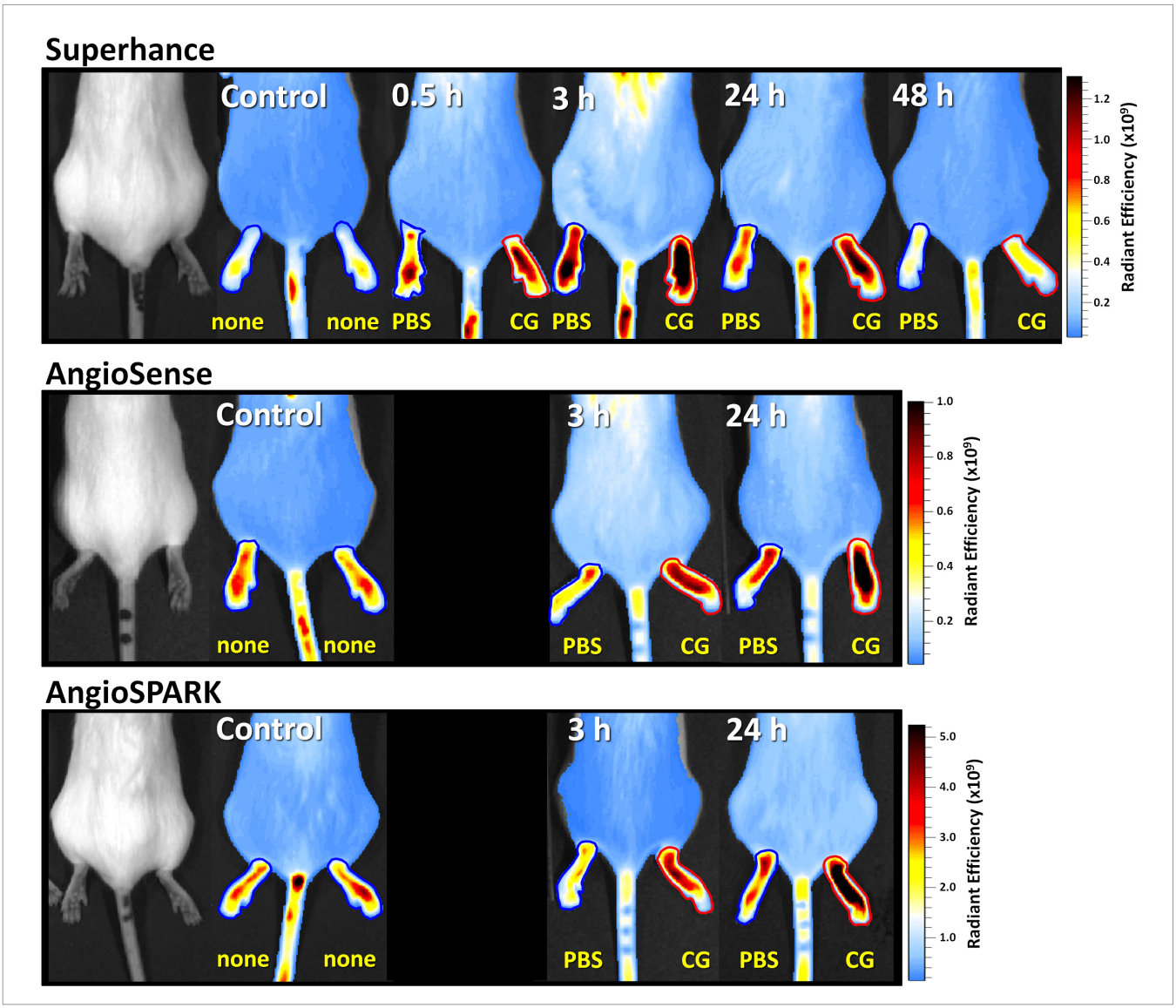

Figure 7. **Superhance 680, AngioSense 680EX, and AngioSPARK 680 paw imaging kinetics.** Representative BALB/c mice are shown that were injected in the right footpad 30 ul of 1% Carrageenan (CG), and in the left footpad with 30 ul PBS. Imaging probes were injected 2 h later and mouse paws were imaged 3 h, 24 h, and 48 h post-probe injection for quantification of fluorescence in inflamed paws, PBS paws, and uninjected mouse controls. Regions of interest (ROI) for tumor (red) and control (blue) paws are indicated.

Edema Quantification

Although only three mice per group were used to illustrate vascular probe performance, statistical significance was achieved in comparison to control paws with all three probes (Figure 8). Background subtraction during analysis (Figure 8B) is important in order to appropriately interpret the magnitude of carrageenan-induced edema without including non-specific signal circulating through the paw or accumulating due to minor tissue damage from a needle stick. For this reason, background correction was performed using mice which were not injected but received probes for imaging at the relevant time points. Thus, background correction accurately portrays paw signal due to needle-stick + PBS alone as well as to injection of carrageenan as an irritant.

Superhance was ideal for detecting and measuring edema at 3 h, the peak of the neutrophilia-induced edema, achieving a corrected inflammation to background ratio of ~6.0. AngioSense and AngioSPARK probes offered optimal detection of edema in inflamed paws at 24 h, with corrected inflammation to background ratios of 4.4 and 2.9, respectively.

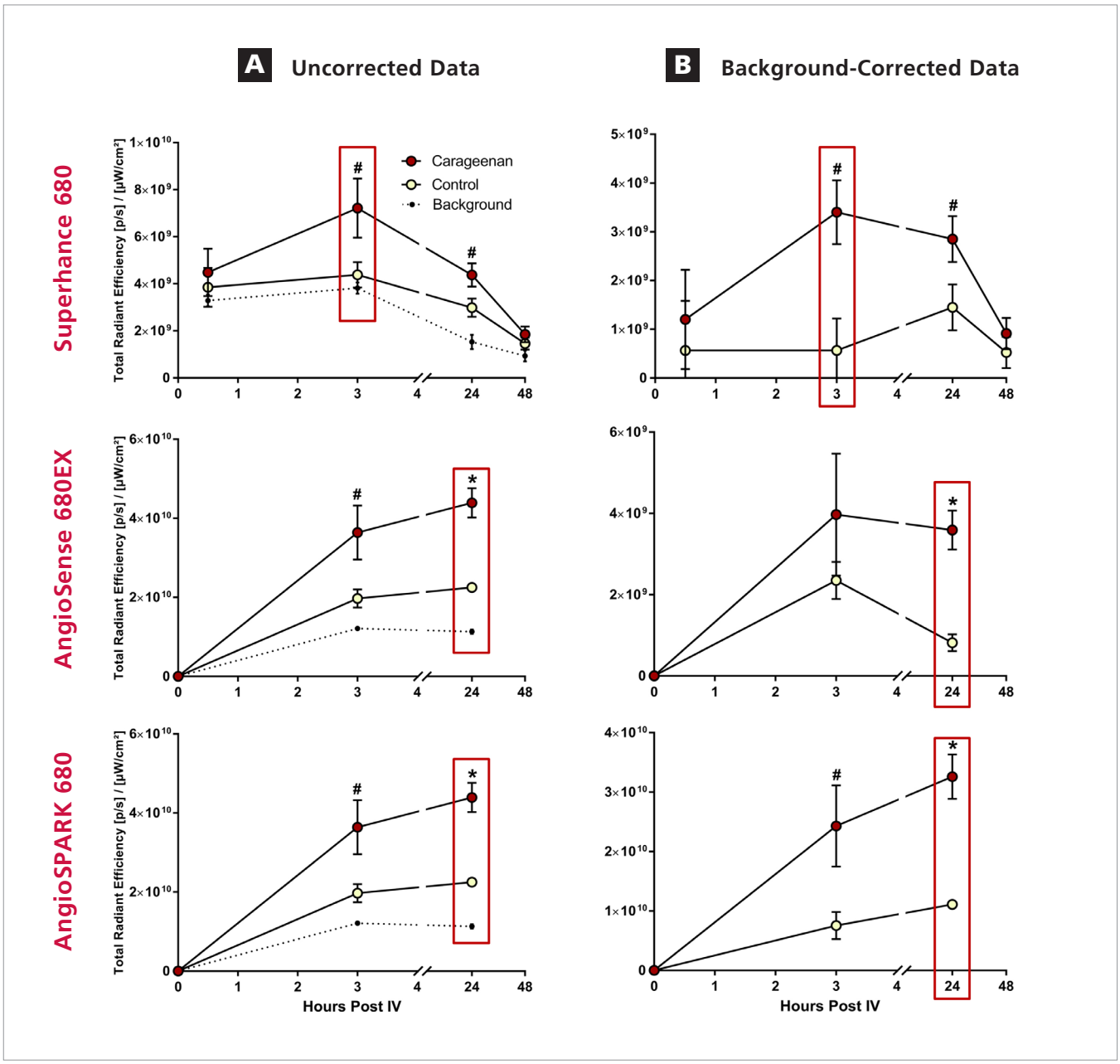

Figure 8. **Quantification and analysis of vascular leak imaging .** (A) Uncorrected and (B) background-corrected data are shown for Superhance 680 (upper), AngioSense 680EX (middle), and AngioSPARK 680 (lower). Statistical differences between CG and PBS paws were assessed by Student's t test at each individual time point.

Conclusions

Superhance 680, AngioSense 680EX, and AngioSPARK 680 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 probes in tumor vascular leak and paw edema imaging as well as including supportive summaries from arthritis and intravital microscopy studies. All three probes provide good tumor definition, with Superhance affording earlier imaging opportunities, albeit with somewhat lower signal. AngioSense generally provides the greatest signal to background, and AngioSPARK shows the longest lasting signal in 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 that have been explored, including asthma, COPD, arthritis, peritonitis, wound healing, graft-versus-host disease, blood-brain-barrier breakdown, and organ failure.

Table 2. **Summary of probes and applications.** An overview of results from this application note, as well as two other key applications, is represented, including practical information regarding optimal imaging/washout time and response to treatments (from internal and external, published and unpublished, research). Performance is based on a subjective scale (+, ++, +++) that takes into account the signal intensity, kinetics, background, washout, and general utility.

	Superhance 680	AngioSense 680EX	AngioSPARK 680
Tumor Imaging	+	+++	++
-Tumor Imaging Time	3-24 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
-Steroid Response	+++	+++	++
Arthritis Imaging	*nd*	+++	+++
-Tissue Imaging Time	*nd*	24 h	24 h
-Tissue Washout	*nd*	144 h	> 192 h
-Treatment Response	*nd*	++	*nd*
Intravital Microscopy	+	+++	+++
-Optimal Imaging Time	5-15 min	5-60 min	5 min – 3 h
-Treatment Response	*nd*	+++	+++

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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

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