IVISense™
FLUORESCENT PROBE
SELECTOR GUIDE

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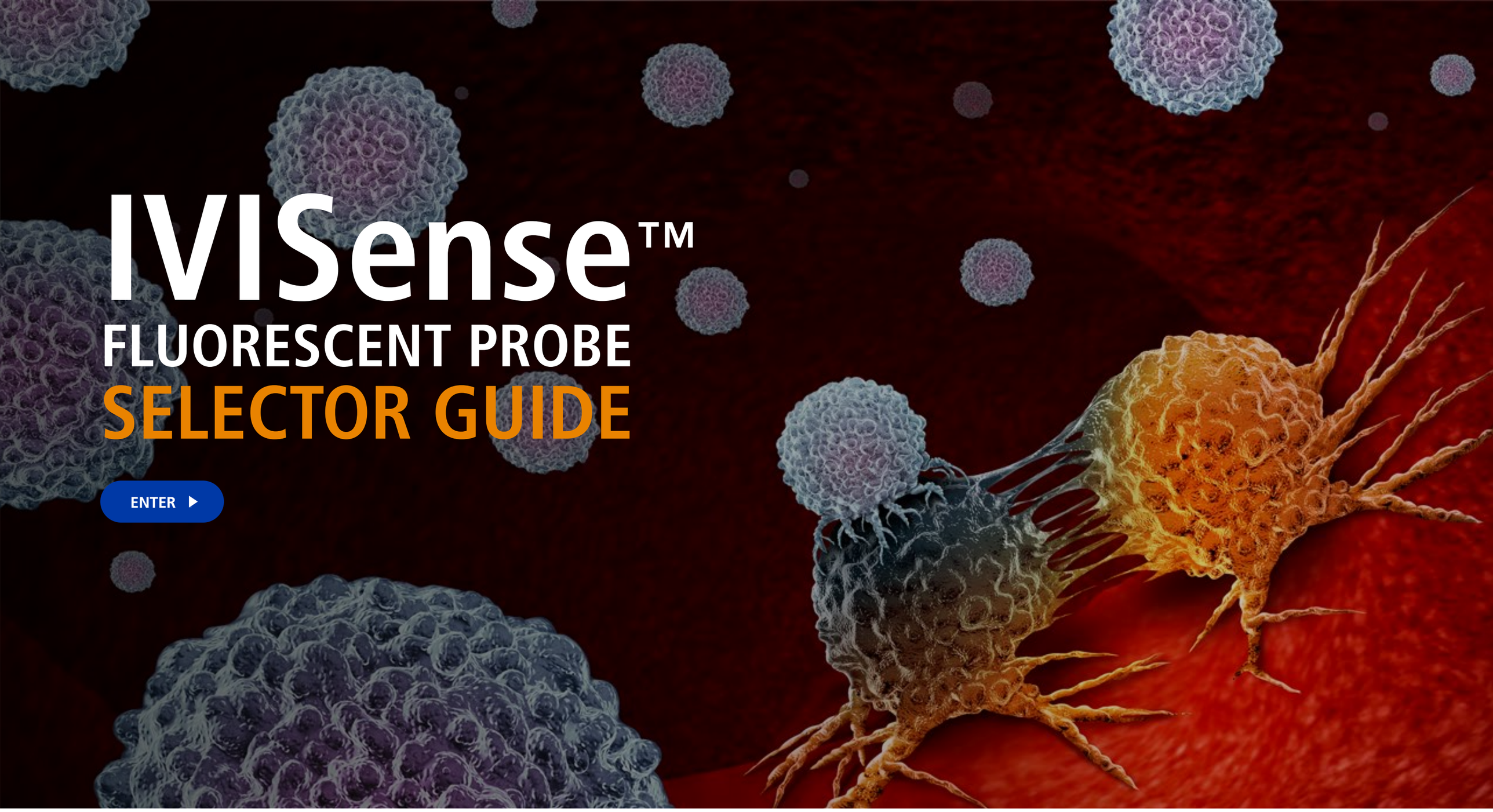

Oncology Series

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REAGENTS DESIGNED FOR YOUR *IN VIVO* IMAGING RESEARCH IS HOW PRECLINICAL ADVANCEMENTS ARE MADE.

ABOUT THIS GUIDE

The goal of *in vivo* fluorescence molecular imaging is to enable non-invasive visualization and quantification of cellular and biological functioning to better understand and characterize disease processes and treatment effects earlier within the context of a biological system.

This can be achieved using biologically specific, validated fluorescent probes. In cancer research, well-defined fluorescent probes can be used to detect specific oncological biomarkers and protease activity, as well as drive cancer therapies.

It's essential to understand how a probe's structure, size, specificity, and physiochemical properties can significantly impact its pharmacokinetics, biodistribution, and metabolic clearance characteristics. These properties, designed and fine-tuned to best suit a certain biology and optimal imaging time point, must be carefully considered in relation to the biological and temporal characteristics of one's model.

With an emphasis on proper probe selection and careful study design, non-invasive assessment of *in vivo* biological readouts can be maximized to detect early indicators of standard endpoints and realize more meaningful research results.

This selector guide for IVISense™ fluorescent probes is a powerful tool to help advance your oncology research. By matching probe properties to specific biology and biomarker characteristics, you can better understand how imaging and quantification of early biological changes associated with disease development, therapeutic efficacy, and drug-induced tissue changes can be realized.

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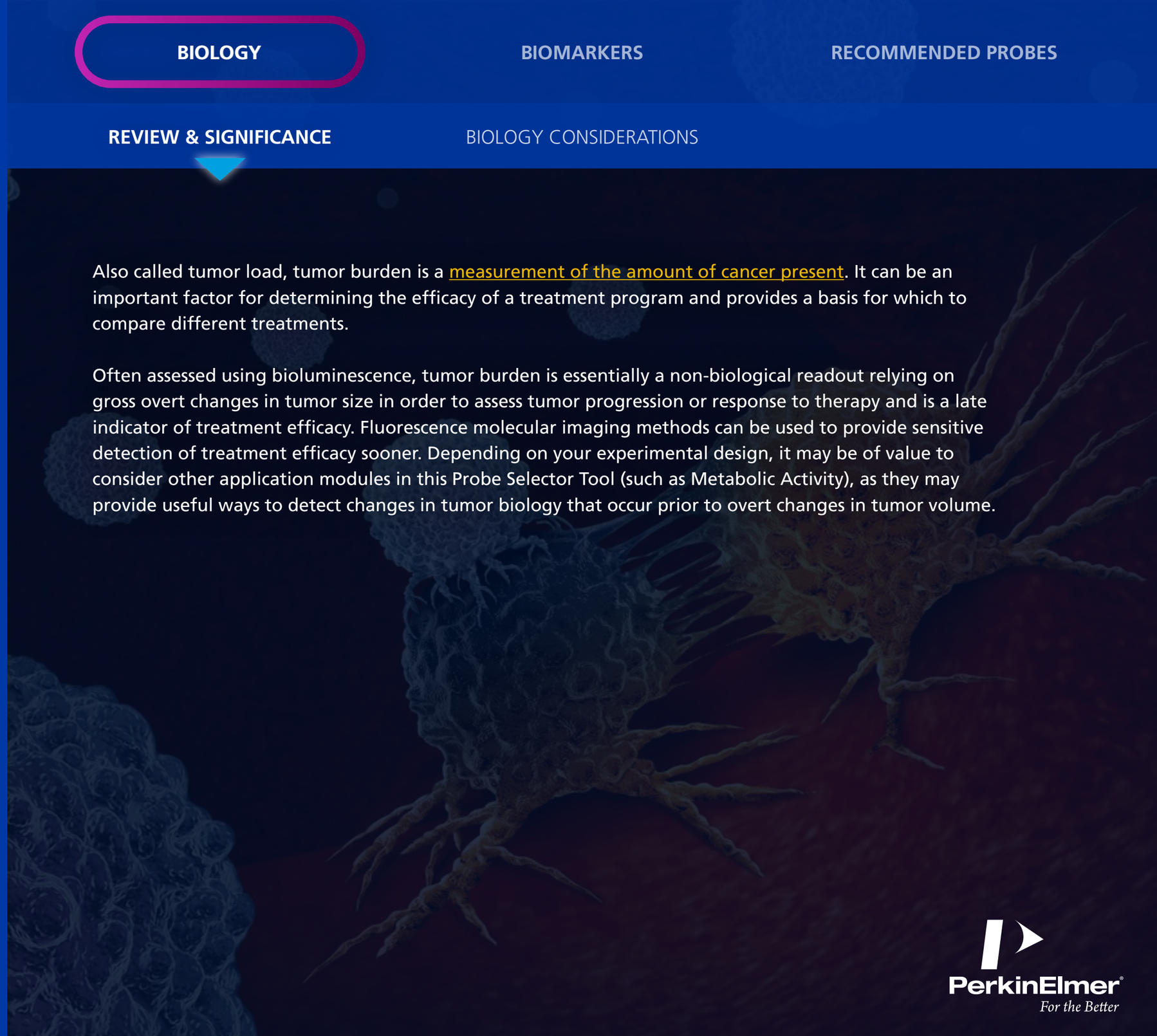
Also called tumor load, tumor burden is a measurement of the amount of cancer present. It can be an important factor for determining the efficacy of a treatment program and provides a basis for which to compare different treatments.

Often assessed using bioluminescence, tumor burden is essentially a non-biological readout relying on gross overt changes in tumor size in order to assess tumor progression or response to therapy and is a late indicator of treatment efficacy. Fluorescence molecular imaging methods can be used to provide sensitive detection of treatment efficacy sooner. Depending on your experimental design, it may be of value to consider other application modules in this Probe Selector Tool (such as Metabolic Activity), as they may provide useful ways to detect changes in tumor biology that occur prior to overt changes in tumor volume.



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MEASUREMENT OF THE AMOUNT OF CANCER PRESENT

Tumor burden is a calculated volume that can be expressed in mm^3. Total tumor burden is the sum of all measured tumor volumes. Traditional models rely on *ex vivo* measurements to obtain tumor volume, or on caliper measurements of width and length to assess *in vivo* longitudinal tumor burden, focusing on changes in shape or size of subcutaneous tumors. However, relying on gross measurement of tumor size to assess treatment efficacy can be misleading, as infiltrating inflammatory cells, calcified parts, and necrotic tissue may obscure treatment effects on viable tumor tissue. Imaging modalities such as optical, microCT or ultrasound are more reliable options for evaluating tumor burden, especially for deep tumor measurement where *in vivo* caliper measurements are not possible.

In the case of optical imaging, bioluminescent imaging (BLI) results generally corroborate conventional caliper measurements quite well, but importantly BLI also takes into account areas of the tumor that may be fibrotic or necrotic. This makes BLI more accurate in measuring viable tumor mass. However, 2D BLI can be quantitatively variable for deep tumors due to attenuation of signal when depth of the signal varies between subjects. Optical tomographic (3D imaging) methods, including both bioluminescence and fluorescence, not only enable an adjusted view of intensity according to its depth and provides better anatomical context for scientific interpretation, but can also be used to achieve more accurate quantification for calculating tumor volume.

Fluorescent probes such as those described in the recommended probes section, or luciferin substrate, can be used effectively with PerkinElmer's IVIS® tomographic (3D) optical imaging systems for accurate longitudinal tumor burden assessment.

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TUMOR SIZE AND LOCATION

Large Tumors: Palpable, well-established tumors can be imaged using the fluorescent agents described in the recommended probes section.

Small Tumors: Detection of tumors smaller than 100 mm^3 is possible but is dependent on tumor location. Tumors located distal from the route of metabolism (the region with the greatest background signal) will be more clearly detected due to the reduced background signal.

PDX TUMORS AND VASCULARITY

As transfection of patient derived xenograft (PDX) tumors with a luc construct is difficult and generally considered undesirable, fluorescence is an appropriate imaging method, and a variety of imaging probes, specific for different aspects of tumor biology, are available to assess growth and response to treatment.

When planning a tumor imaging strategy, consider the vascularization status of the tumor. Vascularization is required for the survival of implanted tumors, but this may take some time to develop, and fluorescent probes may not be as effective immediately following implantation. One approach to consider for early monitoring of tumor implantation is to label with IVISense 680 Cell Labeling Dye. This will allow the tissue surface to be labeled and may provide useful information early on regarding localization and efficiency of implantation (though it will not assess the viability of the tissue).

Once tumor growth is established, fluorescent probes are very effective in assessing growth, metabolism, protease activity, inflammation, cell death, hypoxia, and other important aspects of tumor biology.

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PROBE	BIOMARKER SPECIFICITY	ROUTE OF METABOLISM / BACKGROUND TISSUES
IVISense Cat B 680 FAST	Cathepsin B	Salivary Glands > Liver, Kidneys
IVISense Cat B 750 FAST	Cathepsin B	Salivary Glands > Liver, Kidneys
IVISense MMP 645 FAST	Matrix Metalloproteinase family	Liver > Kidneys
IVISense MMP 680	Matrix Metalloproteinase family	Liver
IVISense MMP 750 FAST	Matrix Metalloproteinase family	Liver > Kidneys
IVISense Pan Cathepsin 680	Cathepsin protease family	Liver
IVISense Pan Cathepsin 750	Cathepsin protease family	Low liver, intestine
IVISense Pan Cathepsin 750 FAST	Cathepsin protease family	Low liver, bladder
IVISense Integrin Receptor 645	αvβ3 Integrin	Bladder, Kidneys
IVISense Integrin Receptor 680	αvβ3 Integrin	Kidneys, Intestines
IVISense Integrin Receptor 750	αvβ3 Integrin	Kidneys

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IVISense 680 fluorescent cell labelling dye is a water soluble dye that enables a simple and easy method for labelling cell membranes. IVISsense 680 cell labeling dye is specially formulated for excellent solubility in the absence of organic solvents such as ethanol or DMSO. The agent intercalates into cell membranes, providing a bright signal by which cells can be tracked *in vivo*.

REQUIREMENTS FOR EFFECTIVE CELL LABELING	IVISENSE 680 CELL LABELING DYE CHARACTERISTICS
Uniform cell labeling	Highly soluble in aqueous solution - does not need to transition from organic to aqueous solution upon addition to the cell suspension, allowing quick dispersion into cell medium and uniform cell labeling
Long cellular residence time	Signal is retained in dividing cells in vitro for more than 7 days
Minimal impact on cell viability and function	Does not use DMSO or ethanol, known to adversely affect cellular function
Near infrared wavelength for optimal light penetration of tissue	Fluorescence emission: 696 nm
Capable of labeling broad range of cells	Effective longitudinal in vivo labeling of: stem cells, T cells, macrophages, and many other cell types

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MMP

Matrix metalloproteases (MMPs) are calcium-dependent, zinc-containing endopeptidases that play a variety of roles in tissue remodeling by degrading the extracellular matrix. Present in healthy biological tissues, they are activated extracellularly by multiple levels of control that strictly regulate their activation. In models of disease, MMP activation can go unchecked, potentially wreaking havoc on essential structural proteins. In cancer models, MMPs tend to localize in tumor margins, playing an important role in tumor metastasis.

The ability to differentiate between active and inactive MMPs at the site of disease, and not just detect the presence of MMPs in general, can reveal distinctive local biology as well as assess therapeutic efficacy of specific drugs prior to overt biological changes. IVISense MMP fluorescent probes are able to detect a broad range of active MMPs including MMP 2, 3, 7, 9, 12, and 13, to track tumor progression, or evaluate the potential therapeutic efficacy of drugs targeting the underlying mechanisms involved.

CATHEPSIN | αvβ3 INTEGRIN

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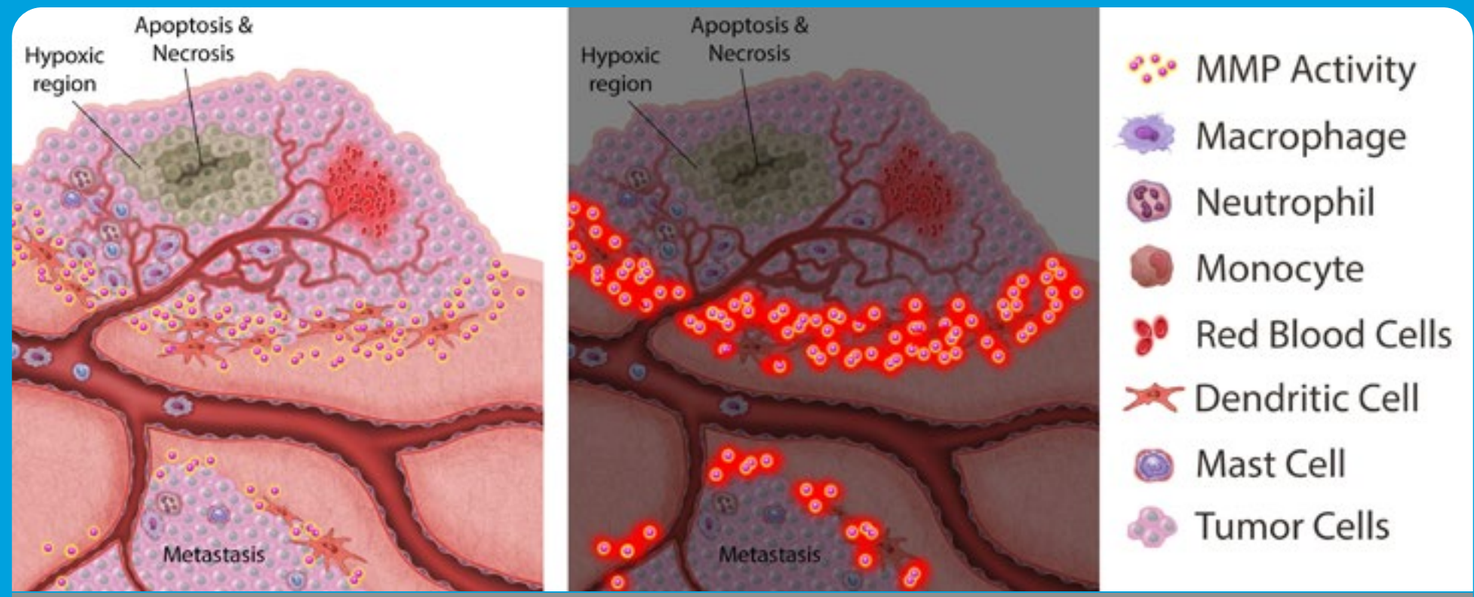

Matrix metalloproteases are active in regions of tissue remodeling, in this case highly expressed and localized at the invasive tumor margins. IVISense MMP fluorescent activation is represented in the figure on the right.

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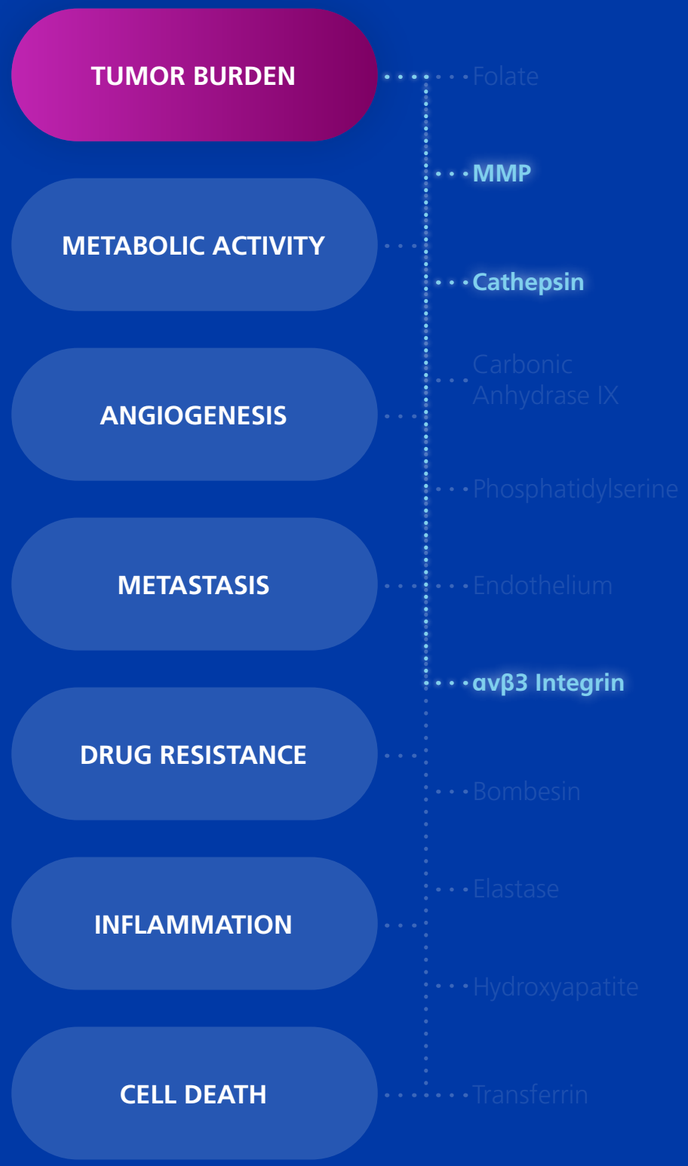

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MMP | CATHEPSIN | αvβ3 INTEGRIN

Associated with tumor aggression, cathepsins are a family of proteases that are pivotal components of signaling pathways which can enhance cancer cell growth and inflammation. Within the human lysosomal cysteine cathepsin family, there are 11 members, including cathepsin B, L, S, and Plasmin. As a family, cysteine cathepsins are involved in the response to anticancer therapy within the tumor microenvironment and play crucial roles in the development of resistance to therapeutics. In particular, cathepsin B expression correlates with the invasive and metastatic capabilities of many tumors, such as in breast cancer where high expression levels of cathepsin B have been linked to highly aggressive tumors. Cathepsin B is also upregulated in a variety of inflammatory cells (including eosinophils, neutrophils, and macrophages).

For fast, non-invasive *in vivo* imaging of tumor status and progression via protease expression, NIR fluorescent imaging probes such as IVISense Pan Cathepsin and IVISense Cat B are available for evaluating potential anticancer therapies. IVISense Pan Cathepsin imaging probes are versatile activated agents, able to detect and measure the expression and activity of key cancer-associated proteases and can be used to monitor the activity of these proteases in real time. IVISense Cat B fluorescent probes are selectively cleaved by cathepsin B proteinases for specific and rapid detection of tumors associated with cathepsin B activity.

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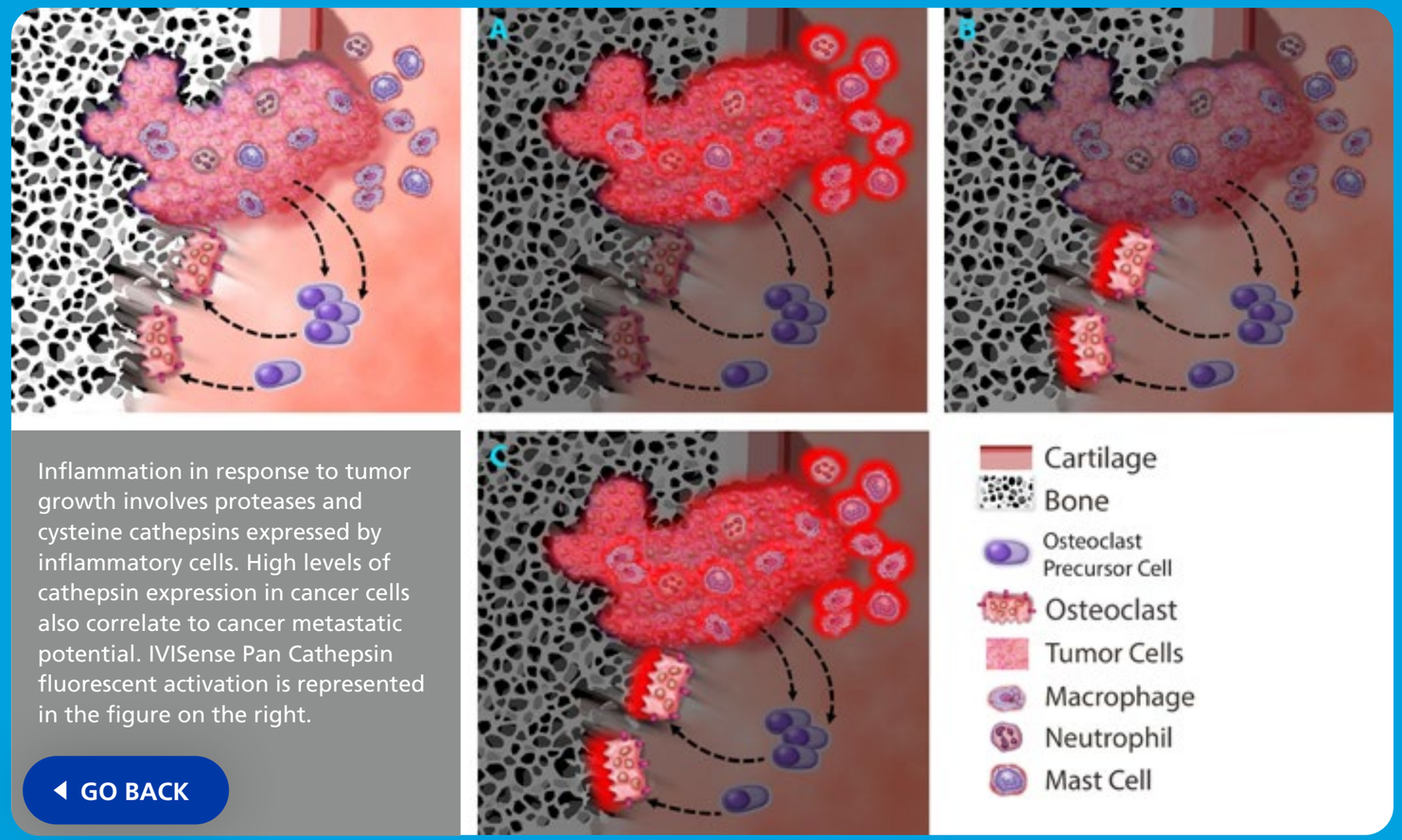

Inflammation in response to tumor growth involves proteases and cysteine cathepsins expressed by inflammatory cells. High levels of cathepsin expression in cancer cells also correlate to cancer metastatic potential. IVISense Pan Cathepsin fluorescent activation is represented in the figure on the right.

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MMP | CATHEPSIN | αvβ3 INTEGRIN

Integrins are cell surface signaling molecules that are essential for regulating communication between cells and their microenvironment. Critically involved in the cells' ability to adhere to the extracellular matrix as well as in cellular migration, they are important to maintaining the integrity of healthy tissue and are abnormally expressed in diseases such as cancer where integrin expression correlates with tumor aggressiveness..

In particular, αvβ3 integrin is significantly upregulated in tumor cells and activated endothelial cells during neoangiogenesis. As a distinct biomarker of tumor burden and progression, αvβ3 integrin can be imaged using IVISense Integrin Receptor NIR fluorescent imaging probe. The IVISense Integrin Receptor probe provides excellent tumor definition with minimal off-target distribution due to its high affinity (Kd = 4.2 nM) and selectivity 5-20x over RDG peptide-based agents. With improved circulation half-life and specificity, IVISense Integrin Receptor probe enables detection and measurement of αvβ3 integrins *in vivo.*

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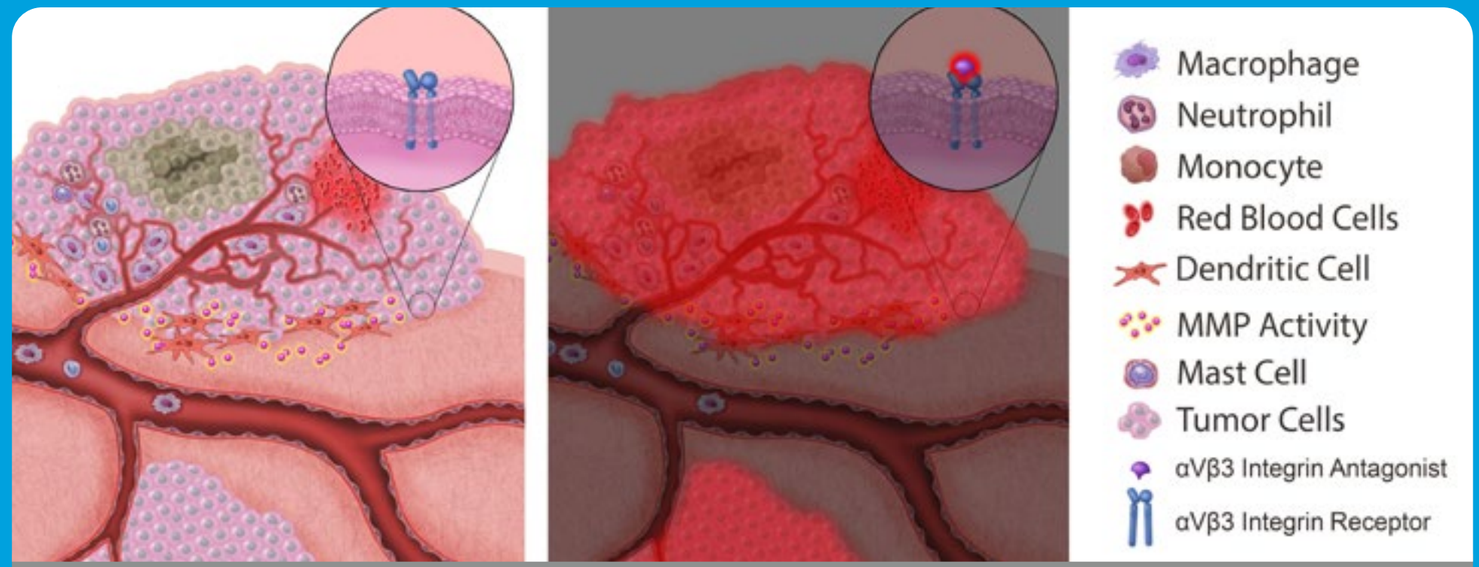
MMP | CATHEPSIN | αvβ3 INTEGRIN



αvβ3 integrin is significantly upregulated in tumor cells and correlates with tumor aggressiveness. IVISense Integrin Receptor fluorescent imaging of fine tumor definition is represented in the figure on the right.

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BIOPROFILING TUMOR BURDEN AND PROTEASE ACTIVITY: IDENTIFYING CHARACTERISTIC DIFFERENCES BETWEEN TUMOR TYPES

Critical to understanding the biology of a model is to know when a particular biomarker is active. Cancer biomarkers may be up- or down-regulated at different times, as different factors may influence their expression during the growth of a tumor. To illustrate the potential of broad tumor profiling, the following case study uses two different tumors imaged with different fluorescent agents. These agents are capable of detecting protease activity or surface receptor expression of markers for assessing tumor cell burden. As shown in the case study, fluorescence quantification with multiple probes can illustrate the importance of giving consideration to characteristic differences in rates of protease expression in tumors for appropriate probe selection, as well as likely prove valuable for the full biological characterization of tumors.

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CASE STUDY: EFFECTS OF PROTEASES ON TUMOR GROWTH AND IMPLANTATION EFFICIENCIES

PerkinElmer's IVISense NIR fluorescent *in vivo* imaging probes are important tools to non-invasively detect and quantify biomarkers such as protease activity (MMPs and cathepsins) and indicators of tumor burden (integrin). Groups of HeLa and 4T1 tumor-bearing mice were injected with two pairs of imaging agents; IVISense MMP 680, IVISense Pan Cathepsin 750, and IVISense Osteo 680 & IVISense Integrin Receptor 750 (IVISense Osteo was used as a control agent not expected to localize appreciably to tumor tissue) to detect relative differences in protease activity (associated with tumor progression and aggression) and integrin expression (general tumor burden marker) in HeLa and 4T1 tumors.

Fluorescence quantification of *in vivo* imaging probes shows that the two different tumor types were quite similar in IVISense Integrin Receptor, indicating similar levels of integrin receptor expression. In contrast, HeLa cells were 50-70% lower in protease activity as assessed by IVISense MMP and IVISense Pan Cathepsin imaging. This lower protease activity in HeLa cells may account for the slower initial growth rate of HeLa tumors (see graph on the right), suggesting the importance of proteases for growth early on, following the implantation of cells. At later time points, the HeLa tumors rapidly achieve the size of the 4T1 tumors.

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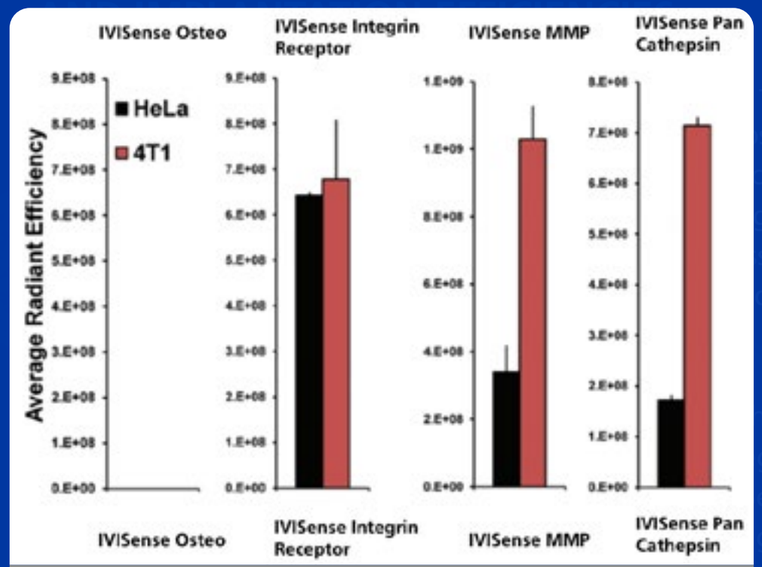
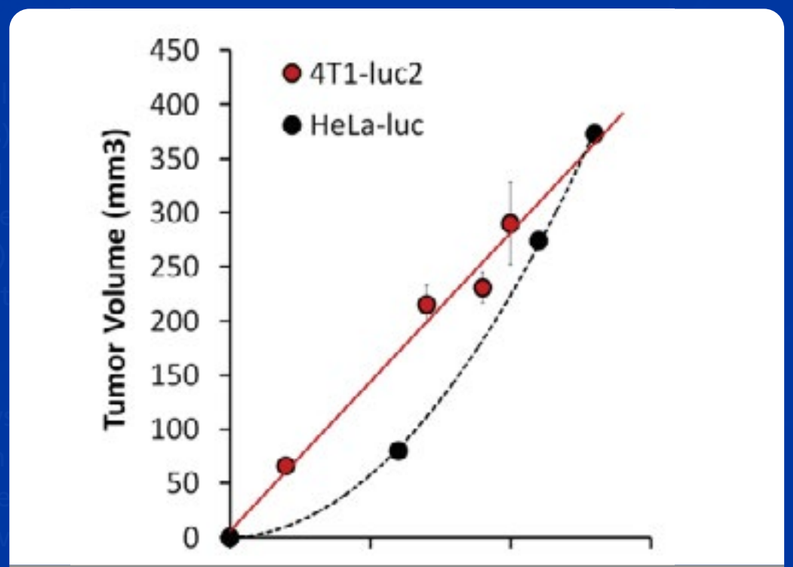


Fluorescence Quantification of *in vivo* imaging probes in HeLa and 4T1 cell lines Imaged on the IVIS® Spectrum optical imaging system.



Tumor Growth Rates. Caliper measurements of 4T1-luc2 and HeLa-luc tumors were measured over time to assess growth kinetics. Length and width measurements were used to calculate tumor volumes in mm^3.

Application Note: Peterson et al., Multiplex 2D Imaging of NIR Molecular Imaging Agents on the IVIS® SpectrumCT and FMT® 4000.

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BY CHARACTERIZATION | BY APPLICATION

IVISense Probe	MW (g/mol)	Specificity	*In Vivo* Biology Use	Mouse Dose (25 g)	Sold As (Unit Size)	Optimal Imaging Time (h)
✂ Cat B 680 FAST	33,000	Cathepsin B	Selectively cleaved by cathepsin B proteinases upregulated in tumors and inflammatory cells.	2 nmol	24 nmol	6-24 h
✂ Cat B 750 FAST	23,000			4 nmol	48 nmol	6-24 h
✂ MMP 645 FAST	43,000	Matrix Metalloprotease	Cleaved by multiple metalloproteinases associated with many disease areas including cancer, inflammation, and cardiovascular disease.	4 nmol	48 nmol	24 h (6-24)
✂ MMP 680	~450,000			2 nmol	20 nmol	24 h (24-36)
✂ MMP 750 FAST	43,000			2 nmol	24 nmol	24 h (12-24)
✂ Pan Cathepsin 680	~400,000	Cathepsin proteases	Pan-cathepsin activatable agent that detects abnormal upregulation of cathepsin secretion associated with cancer and inflammation.	2 nmol	20 nmol	24 h (24-48)
✂ Pan Cathepsin 750	~450,000			2 nmol	24 nmol	24 h
✂ Pan Cathepsin 750 FAST	22,500			4 nmol	48 nmol	6-24 h
⌖ Integrin Receptor 645	1,250	αVβ3 Integrin	Detection of integrin αvβ3 expression in tumors and neovasculature.	2 nmol	24 nmol	6-24 h
⌖ Integrin Receptor 680	1,432			2 nmol	24 nmol	24 h
⌖ Integrin Receptor 750	1,278			2 nmol	24 nmol	24 h

✂ Activatable probes are optically silent upon injection and become highly fluorescent following protease-mediated activation. Activatable FAST agents are designed with a novel small molecule architecture that confers an accelerated pharmacokinetic profile with earlier imaging time points.

⌖ Targeted probes actively target and bind to distinct biomarkers with highly specific targeting to key biological mechanisms.

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BY CHARACTERIZATION | BY APPLICATION

IVISense	Cat B 680 FAST Cat B 750 FAST	MMP 680	MMP 645 FAST MMP 750 FAST	Pan Cathepsin 680 Pan Cathepsin 750	Pan Cathepsin 750 FAST	Integrin Receptor 645 Integrin Receptor 680 Integrin Receptor 750
Biomarker specificity	Cathepsin B	Matrix Metalloprotease family proteases	Matrix Metalloprotease family proteases	Cathepsin family proteases	Cathepsin family proteases	αvβ3 Integrin
Applications						
Early Tumor Assessment	+++	+++	+++	+++	+++	++++
Metastasis Imaging	++	+/- due to liver interference	++	+/- PC680 (liver) ++ PC750	++	++
Image Tumor Vascularity	-	-	-	-	-	+++
Assessing Treatment Efficacy	+++	+++	+++	+++	+++	+++
Probe Characteristics						
Imaging Time Post Probe Injection	6-24 hr	24 hr (24-36)	24 hr (6-24) 24 hr (12-24)	24 hr (24-48)	6-24 hr	6-24 hr 24 hr 24 hr
Tumor Washout	3 days	6-7 days	6-7 days	6-7 days	3 days	6-7 days 14 days 4-6 days

Summary of probes and applications. An overview of results, 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.

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Cancer is highly adept at hijacking cellular pathways to gain a biological advantage. Metabolic reprogramming exploits the system of biochemical processes within cells, altering pathways to meet the increased demand for energy, synthesis, and proliferation that is characteristic of tumor growth.

Biomarkers of these altered pathways offer opportunities to study not only tumor progression, but also to specifically assess therapies that target critical pathways in tumor metabolism. Detecting biomarker fluctuations by *in vivo* fluorescence imaging can provide an early indication of changes in tumor metabolism and growth, prior to overt changes in tumor burden as detected by traditional caliper assessment or standard bioluminescence.

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IMAGING HEPATIC TUMORS

Iron is necessary for cellular metabolism in cells throughout the body. In healthy tissue, cellular iron homeostasis is maintained in part by transferrin receptors. In tumor cells, increased metabolic demand for iron requires the number of transferrin receptors on the cell surface to increase. Therefore, increased expression of transferrin receptors can be exploited as a biomarker of tumor metabolism and effectively targeted for fluorescent *in vivo* imaging.

When developing an imaging strategy for a tumor in the liver, the main site of iron storage in the body, it is important to choose a probe with a non-interfering route of metabolism (the region with the greatest background signal). As some probes are metabolized through the liver, choosing a probe that metabolizes away from the liver will allow for better detection due to reduced background signal.

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INFLAMMATION
CELL DEATH

Folate
MMP
Cathepsin
Carbonic Anhydrase IX
Phosphatidylserine
Endothelium
αvβ3 Integrin
Bombesin
Elastase
Hydroxyapatite
Transferrin

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MEASUREMENT OF THE AMOUNT OF CANCER PRESENT

PROBE*	BIOMARKER SPECIFICITY	ROUTE OF METABOLISM / BACKGROUND TISSUES
IVISense Folate Receptor 680	Folate receptor	Kidney
IVISense Bombesin Receptor 680	Bombesin receptor	Pancreas, kidney
IVISense Transferrin Receptor 750	Transferrin receptor	Liver, kidney

*Other probes , such as IVISense Pan Cathepsin 750 FAST & 750, IVISense MMP 750 FAST, and IVISense Integrin Receptor, also metabolize away from the liver, and are capable of imaging hepatic tumors for biological applications other than detecting biomarkers of metabolic activity (for example, detecting biomarkers of inflammation or tumor burden and progression). See the Fluorescent Agents Reference Table in the Resources section for a full list of probes and their specificity, routes of metabolism, and more.

TUMOR BURDEN
- Folate

METABOLIC ACTIVITY
- MMP
- Cathepsin
- Carbonic Anhydrase IX

ANGIOGENESIS
- Phosphatidylserine

METASTASIS
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FOLATE

Folate is essential for cellular metabolism and DNA synthesis, methylation, and repair. Rapidly proliferating cancer cells have increased demand for folate to maintain their accelerated DNA synthesis; thus overexpression of folate receptors (FRs) is a recognized biomarker of many tumors, though tumors can differ in their dependence on folate metabolism.

The ability to quantify FR expression to distinguish between high and low FR expressing tumors is important for the efficient delivery of chemotherapeutic agents, drug carriers, photosensitizers, and diagnostic reporters. IVISense Folate Receptor NIR fluorescent probe enables the non-invasive visualization and quantification of FR expression on highly metabolic cancer cells.

VIEW IMAGE ▶

BOMBESIN | TRANSFERRIN

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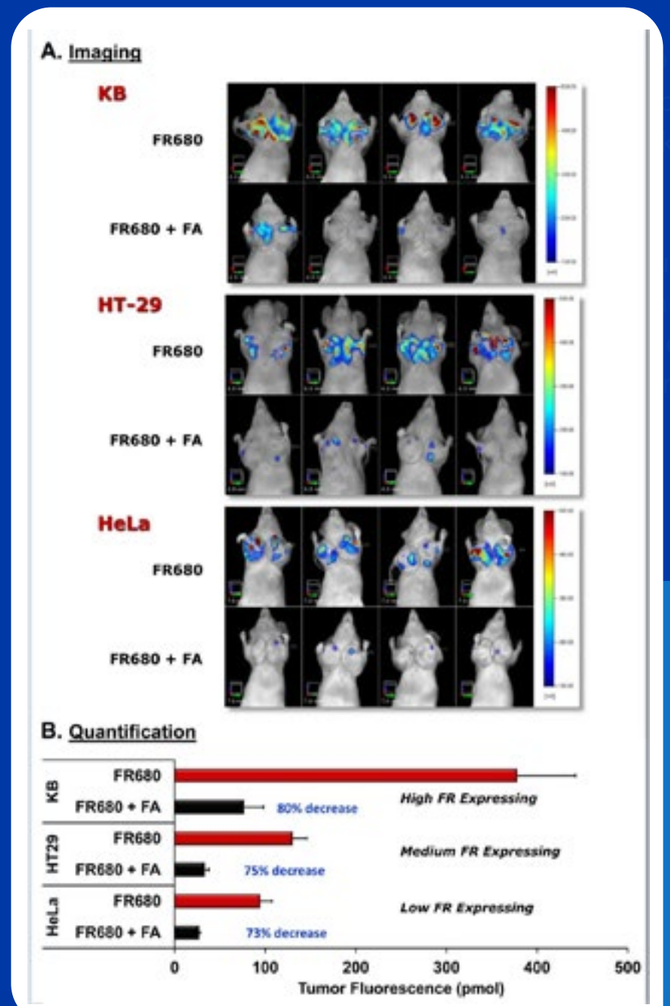
FOLATE RECEPTOR (FR) EXPRESSION IN CANCER



Determining tumor dependence on folate metabolism can be important because folic acid (the synthetic form of folate) has been successfully exploited as a cancer specific targeting moiety for the efficient delivery of various treatments. Thus the level of FR expression for a given tumor is critical to the success of these treatments, since weak FR-expressing cancers will not respond well to folate-targeted therapies.

Below demonstrates how IVISense Folate Receptor 680 (FR680) probe was used to determine high, medium, and low FR expression levels in three different cancer cell lines.

KB, HeLa or HT-29 tumor bearing mice were injected with FR680 in the absence or presence of 100x excess folic acid and imaged tomographically 4h later.

A. Shown are 4 representative mice per group.
B. Quantification of FR680 signal in tumors. Results show folate-specific blockade of FR680 accumulation in the tumors, even in the case of low-expressing tumor lines. This is attributed to the presence of FR-expressing inflammatory cells.

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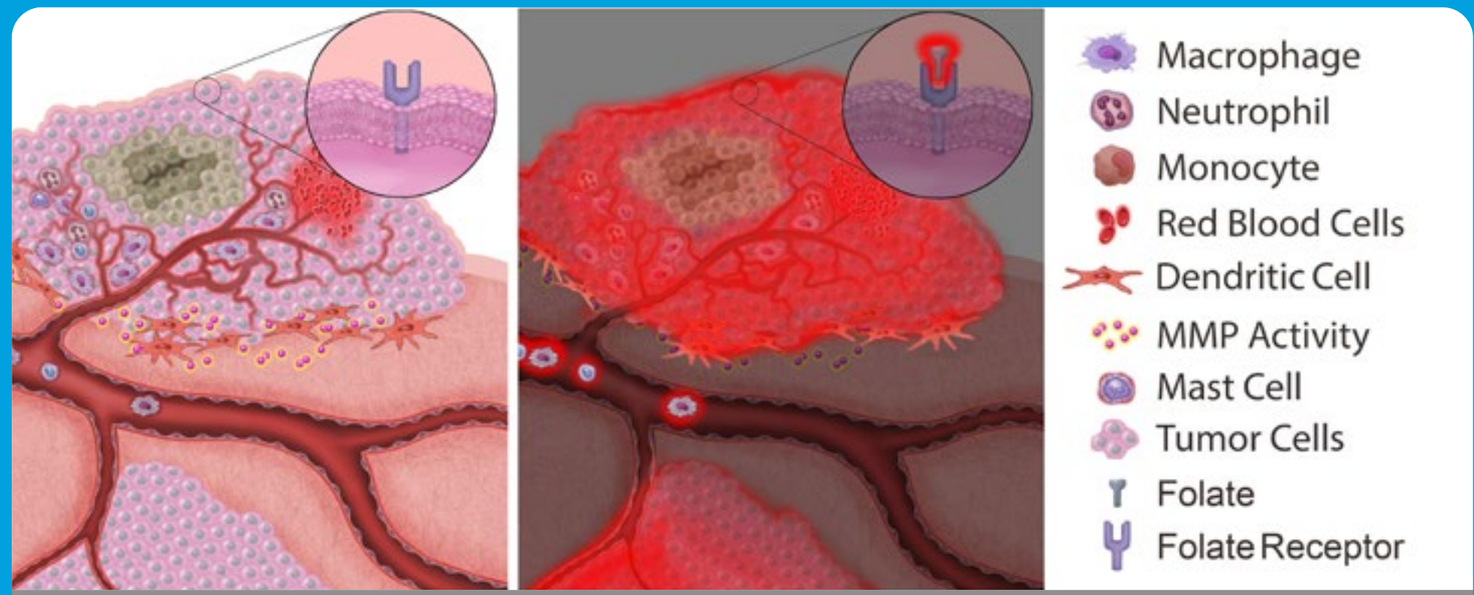
FOLATE | BOMBESIN | TRANSFERRIN



Depending on the tissue of origin, tumors overexpress metabolic receptors that can be used as biomarkers of cancer progression. The image above illustrates surface membrane receptors for folate. The panel on the right illustrates fluorescence for the folate receptor, showing less activity in dead, hypoxic, or poorly vascularized regions, where metabolic activity is reduced.

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FOLATE | BOMBESIN | TRANSFERRIN

Bombesin is a 14-amino acid peptide originally isolated from the European fire-belly toad (*Bombina bombina*). Bombesin-like peptides and bombesin receptors (BBRs) have been the subject of investigation for the past two decades because of their involvement in cancer cell energy metabolism and proliferation. Mammalian bombesin analogs, such as gastrin-releasing peptide (GRP), can promote cell growth, malignant transformation, and tumor differentiation, such that many types of human cancer, including prostate, breast, lung, CNS, gastric, colon, and renal, show upregulated expression of BBRs on the surface of tumor cells.*

As potential targets for drug delivery, further exploration of these receptors is warranted. IVISense Bombesin Receptor NIR fluorescent imaging probe, a novel NIR fluorescent imaging agent, is comprised of a 7-amino acid bombesin peptide analog, an NIR fluorophore, and a pharmacokinetic modifier to improve its plasma availability, and is an ideal probe to target and quantify upregulation of bombesin receptors *in vivo* associated with tumor proliferation.

VIEW IMAGE ▶

*Tseng et al. Fluorescence imaging of bombesin and transferrin receptor expression is comparable to ^{18}F-FDG PET in early detection of sorafenib-induced changes in tumor metabolism. *PLoS ONE* 12(8): 2017. e0182689.

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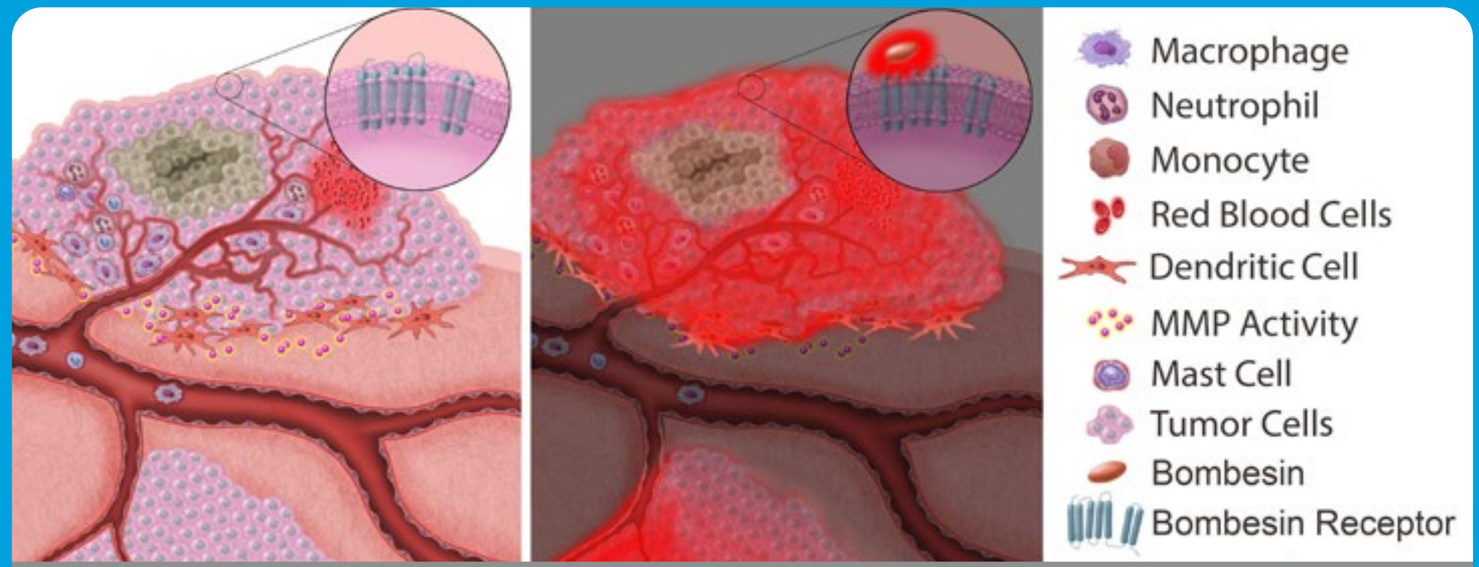
FOLATE | BOMBESIN | TRANSFERRIN



Depending on the tissue of origin, tumors overexpress metabolic receptors that can be used as biomarkers of cancer progression. The image above illustrates surface membrane receptors for bombesin. The panel on the right illustrates fluorescence for the bombesin receptor, showing less activity in dead, hypoxic, or poorly vascularized regions, where metabolic activity is reduced.

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FOLATE | BOMBESIN | TRANSFERRIN

Transferrin is an important serum protein that mediates iron transport into cells via the transferrin receptor in the cell membrane. This function is essential for cell growth and metabolism. Increased transferrin receptor levels are associated with an altered metabolic need for iron, such as in many cancers where increased iron levels are required to support the demands of tumor growth.

Transferrin receptors can serve as a useful biomarker for imaging changes in tumor metabolism and biology prior to overt physical changes, thus making it possible to evaluate potential cancer drugs earlier. IVISense Transferrin Receptor fluorescent probe is compromised of PEGylate recombinant transferrin and a NIR fluorescent reporter, is specific for cell surface transferrin receptors and can be used to detect early effects of drugs designed to inhibit tumor metabolism.

VIEW IMAGE ▶

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TRANSFERRIN RECEPTOR EXPRESSION IN CANCER

Increased iron metabolism is linked to tumor progression as well as drug resistance and immune evasion. Treatment methods may involve decreasing intracellular iron stores, a method currently being studied, or alternatively, iron overload, which is toxic to cells and can cause a form of regulated cell death in cancer growths called ‘ferroptosis’*.

The table below from Brown et al, shows two types of transferrin receptors identified and their relevance in cancer. Iron transport proteins as well as transferrin-based probes like IVISense Transferrin Receptor bind to both transferrin receptors TfR1 and TfR2.

Expression of Iron Metabolism Related Proteins and Relevance to Cancer*

Protein	Sample type	Relevance in cancer
Transferrin receptor 1 (TfR1)	Cell lines	Overexpressed in breast, colon, prostate, leukemia, and esophageal cancer cells.
	Tissue samples	Elevated in esophageal, colon, and lung tumors vs. normal tissues. Expression was elevated with increasing stage of liver cancer and correlated with poor prognosis of glioma and breast cancers.
	Serum	Higher in prostate cancer patients than healthy controls.
Transferrin receptor 2 (TfR2)	Cell lines	Upregulated in ovarian, colon, and glioblastoma cancer cell lines.
	Tissue samples	Expression correlated with high tumor grade, but inversely correlated with prognosis of glioblastoma and leukemia. Expressed in a proportion (~26%) of colon cancers.

*Brown et al. Altered Iron Metabolism and Impact in Cancer Biology, Metastasis, and Immunology. *Front Oncol*. 2020;10:476.

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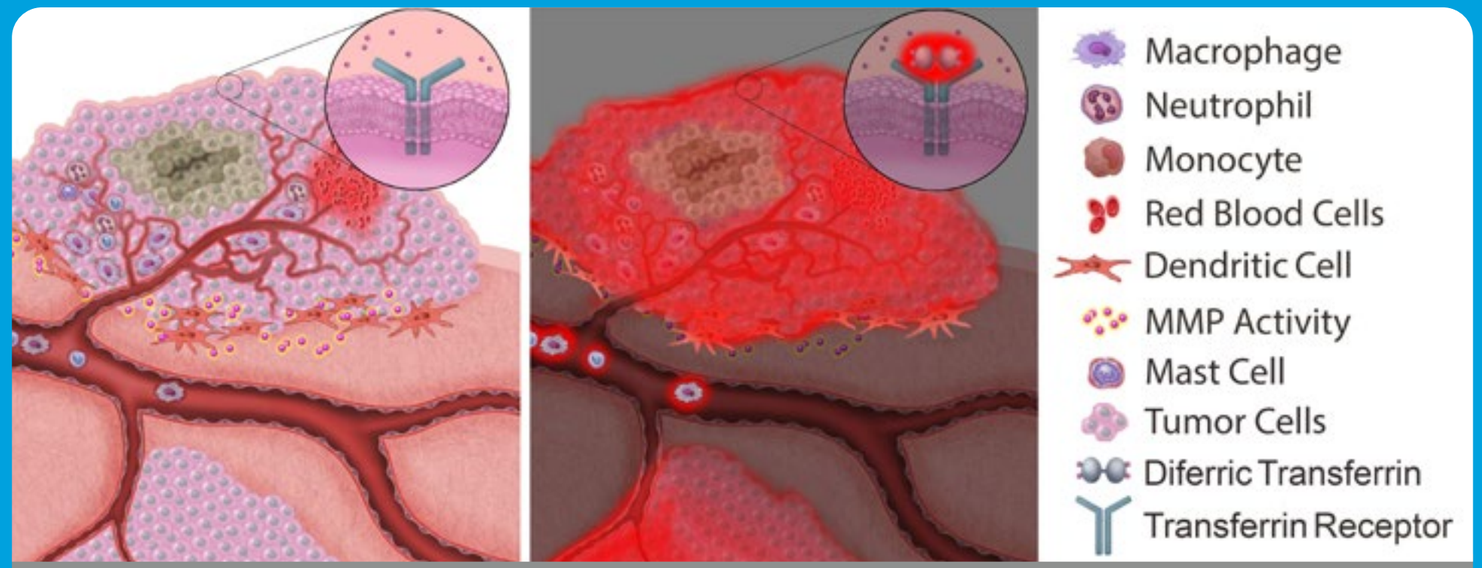

Depending on the tissue of origin, tumors overexpress metabolic receptors that can be used as biomarkers of cancer progression. The image above illustrates surface membrane receptors for Transferrin. The panel on the right illustrates fluorescence for the transferrin receptor, showing less activity in dead, hypoxic, or poorly vascularized regions, where metabolic activity is reduced.

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FOLATE RECEPTOR PROBE UPTAKE AND THE EFFECTS OF INFLAMMATORY CELLS

Folate metabolism occurs in inflammatory cells as well, and folate receptors (FRs) are overexpressed on activated (but not resting or quiescent) macrophages.[1] In drug efficacy studies, where tumor cells may be damaged by the effects of a drug, inflammation will increase in the affected area as a phagocytic response to dead and dying cells. As tumor folate uptake decreases, folate uptake by inflammatory cells in the area may increase, leading to either no change in total FR probe uptake or an increase overall upon treatment. As such, it is important to have a full understanding of the biology of a tumor, as a very inflamed tumor may exhibit paradoxical FR imaging results.

1. Low PS, Henne WA, Doorneweerd DD. Discovery and Development of Folic-Acid-Based Receptor Targeting for Imaging and Therapy of Cancer and Inflammatory Diseases. *Acc Chem Res*. 2008; 41:120-129.

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BEYOND GLUCOSE: OTHER INDICATORS OF ALTERED TUMOR METABOLISM

Called the “Warburg Effect”, glycolysis under aerobic conditions is an important method by which tumors alter their metabolic pathways to support their growth and proliferation.

Although glucose uptake is often the focus when interrogating tumor metabolism, other biomarkers are affected by changes in tumor metabolism and can be exploited as indicators of altered cellular metabolism. Many are fairly ubiquitous biomarkers that are relevant in most if not all cancers. Upregulated to glean nutrients from their environment and provide for their increased metabolic needs, these surface receptors can serve as highly useful biomarkers with remarkable capability as early indicators of tumor treatment efficacy.

FLUORESCENCE IMAGING IS COMPARABLE TO ^{18}F-FDG PET IN EARLY DETECTION OF CHANGES IN TUMOR METABOLISM

In this case study, researchers tested a multiplex optical imaging strategy in a tumor xenograft model to demonstrate how metabolic fluorescence imaging can have remarkable agreement with PET imaging, showing it’s potential in preclinical application as an additional method for detecting drug-induced metabolic changes in tumors.

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Sorafenib treatment of HCT116-luc2 tumors was imaged with bioluminescence, fluorescence, and ^{18}F-FDG PET. Fluorescence imaging used two probes: IVISense Bombesin Receptor 680 (BombesinRSense 680) and IVISense Transferrin Receptor 750 (Transferrin-Vivo 750). Results show the capability of the two fluorescent probes to detect metabolic changes in tumors in the first 3 days after treatment, prior to overt changes in tumor volume, while no overt changes were detected in bioluminescence in the same period. Fluorescent probe detection of early tumor metabolic changes was corroborated by ^{18}F-FDG PET, showing that biomarkers can be used as predictive efficacy markers in fluorescence imaging to indicate pharmacologic response shortly after treatment, and prior to surrogate or clinical endpoints.

This study demonstrated the synergistic use of multimodal imaging to depict a comprehensive picture of the tumor response to drug treatment. More importantly, results indicate the ability of fluorescent imaging probes to detect pharmacologic response prior to surrogate or clinical endpoints. Fluorescent probe detection of metabolic disturbances that occurred within 2-3 days was corroborated by ^{18}F-FDG PET, the gold standard in metabolic imaging.

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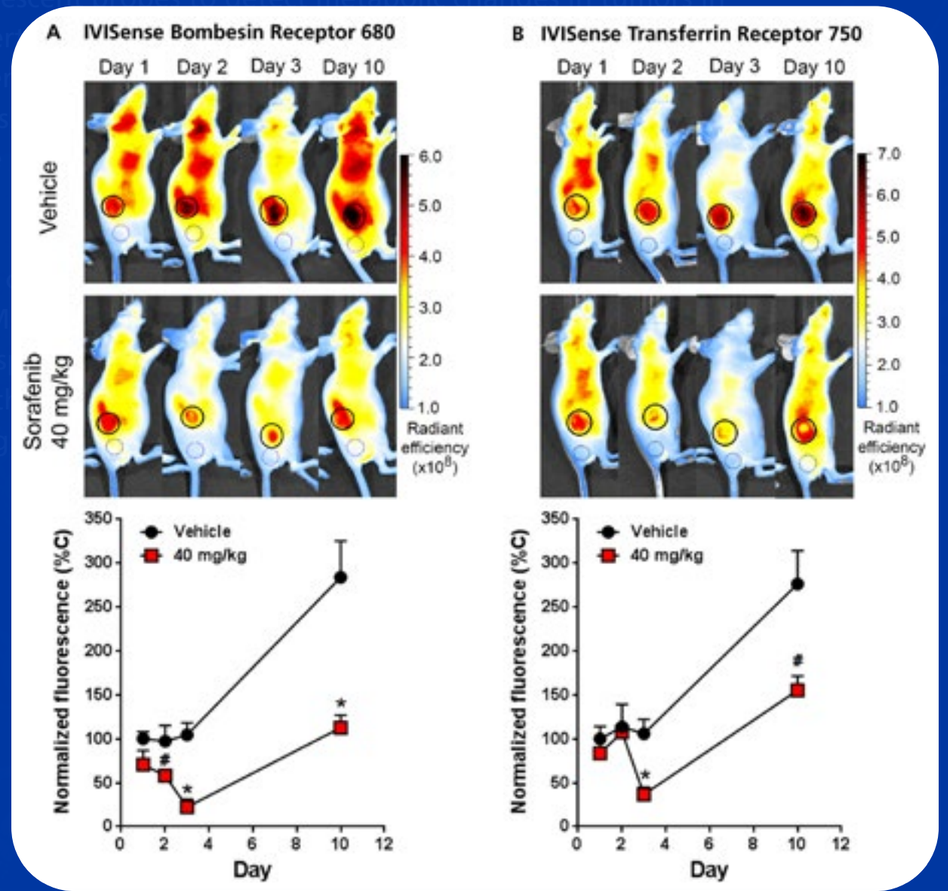
MONITORING SUBTLE TUMOR METABOLIC CHANGES IN RESPONSE TO LOW DOSE SORAFENIB

Metabolic changes in HCT116-luc2 tumors were visualized using IVISense Bombesin Receptor 680 and IVISense Transferrin Receptor 750.

A: Representative mouse images are shown for 2D fluorescence imaging of IVISense Bombesin Receptor 680 (BombesinRSense 680) tumor uptake, with quantitation of tumor fluorescent signal graphed below. Tumor regions of interest (ROI) are represented by solid line circles, and background control ROI are represented by dotted line circles.

B: Representative mice are shown for IVISense Transferrin Receptor 750 (Transferrin-Vivo 750) uptake in tumors, with quantitation of tumor fluorescent signal graphed below.

DIAGRAM 1 | DIAGRAM 2



Peterson, J. et al. Fluorescence imaging of bombesin and transferrin receptor expression is comparable to ^{18}F-FDG PET in early detection of sorafenib-induced changes in tumor metabolism. *PLoS One*. 2017; 12(8): e0182689.

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SUMMARY ANALYSIS OF TUMOR VOLUME: BIOLUMINESCENCE, FLUORESCENCE, AND PET IMAGING RESULTS NORMALIZED FOR COMPARISON

A: Tumor volume

B: BLI signal

C: PET signal

D, E: IVISense Bombesin Receptor 680 and IVISense Transferrin Receptor 750 fluorescence imaging signals respectively. Tumor metabolic changes due to pharmacologic response were detected by fluorescent probe by day 3, corroborated by ^{18}F-FDG PET

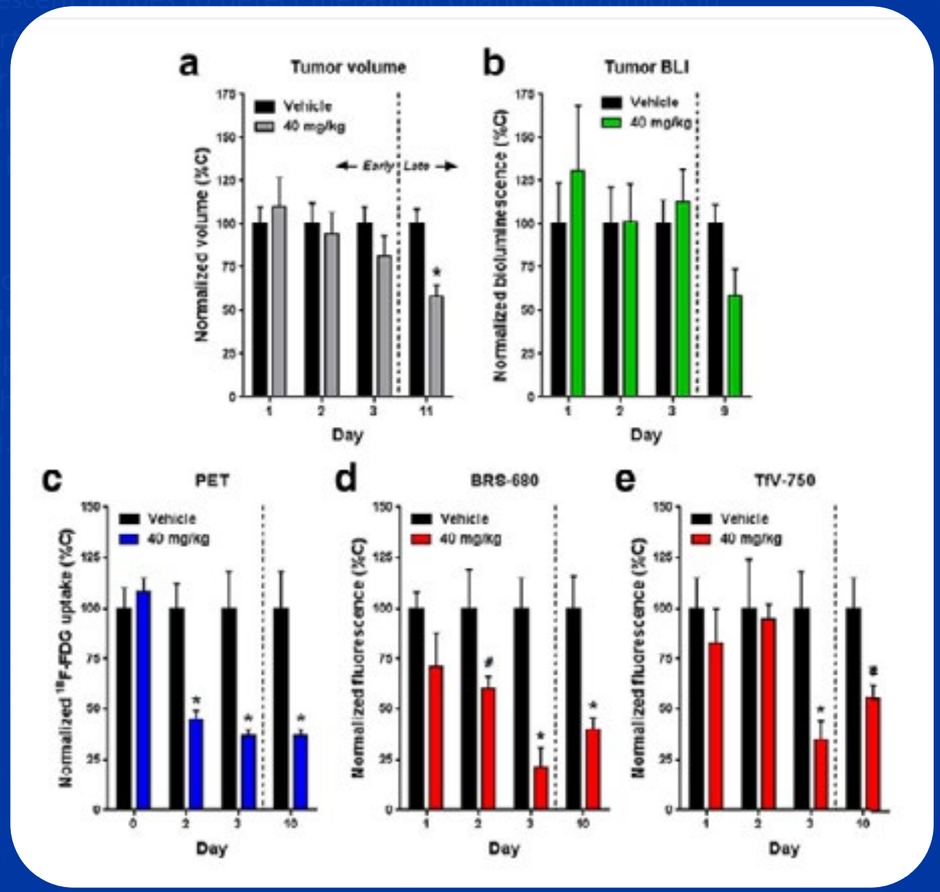

DIAGRAM 1 DIAGRAM 2

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BY CHARACTERIZATION | BIOPROFILING

IVISense Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25 g)	Sold As (Unit Size)	Optimal Imaging Time (h)
Folate Receptor 680	1,606	Folate receptor	Detection of folate receptor protein upregulation for monitoring tumor growth and metabolism.	2 nmol	24 nmol	6 h (6-24)
Bombesin Receptor 680	24,000	Bombesin receptor	Detects upregulation of bombesin receptors on tumor cells associated with increased tumor proliferation.	2 nmol	24 nmol	24 h
Transferrin Receptor 750	106,000	Transferrin receptor	Detects upregulation of transferrin receptors on tumor cells associated with increased tumor metabolism.	2 nmol	24 nmol	24 h (6-24)

Targeted Probes actively target and bind to distinct biomarkers with highly specific targeting to key biological mechanisms.

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BY CHARACTERIZATION | BIOPROFILING

METABOLISM AND THE TUMOR MICROENVIRONMENT

Tumors exist within the context of their microenvironment. In addition to tracking biomarkers of metabolism, the ability to non-invasively measure tumor microenvironment biology can offer a more complete picture of treatment-induced biological changes, very early on prior to over tumor regression.

PerkinElmer offers a fluorescent Tumor Metabolism Panel consisting of a carefully curated collection of probes to detect multiple aspects of tumor biology. Including probes at various wavelengths (680 nm and 750 nm), the panel offers the opportunity for multiplex imaging of appropriate probe combinations to maximize information gained from your research studies.

Tumor Metabolism Panel
Set of Fluorescent Probes Bundled by Application

IVISense Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25g)	Amount (Unit Size)	Optimal Imaging Time (h)	Probe Clearance
Vascular 680	~70,000	Vascularity	Leaks into sites of edema	2 nmol	24 nmol	24 h	6-7 d
MMP 750 FAST	43,000	Matrix Metalloprotease family proteases	Cleaved by multiple metalloproteinases - secreted markers of neutrophils, macrophages, and mast cells	2 nmol	24 nmol	24 h (12-24)	6-7 d
Pan Cathepsin 680	~400,000	Cathepsin family proteases	Images lysosomal marker in a variety of inflammatory cells	2 nmol	20 nmol	24 h (24-48)	6-7 d
Folate Receptor 680	1,606	Folate receptor	Targets marker on inflammatory cells; metabolic marker on tumor cells	2 nmol	24 nmol	6 h (6-24)	6-7 d
Bombesin Receptor 680	24,000	Bombesin receptor	Targets metabolic marker on tumor cells	2 nmol	24 nmol	24 h	6-7 d
Transferrin Receptor 750	106,000	Transferrin receptor	Targets iron metabolism marker on tumor cells	2 nmol	24 nmol	24 (6-24) h	4 d
Annexin-V 750	35,000	Phosphatidyl exposure on cells	Detects surface phosphatidylserine increase on dying cells, Death/apoptosis	100 uL	1 mL	2 h	3 d

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Angiogenesis describes the formation of new blood vessels from pre-existing vessels to meet the demand for nutrients in metabolically active tissue. Tumor angiogenesis refers to the process for establishing vasculature specifically to meet the increased nutrient demands of a growing tumor. Unlike healthy angiogenesis, tumor vasculature can be impaired due to oncogenic mutations in proteins that normally join the endothelial cells together to form the lining of blood vessel walls, resulting in increased vascular permeability. Fluids are able to leak out and pool in surrounding tissues, contributing to tumor metastasis, as well as providing an early indication of tumor progression.

Distinct vascular changes can also characterize inflammation. In acute inflammation, functional changes such as vasodilation and increased vascular permeability are prevalent. These short-term changes are induced by the actions of inflammatory mediators, allowing the appropriate cells, cytokines, and other factors to have access to tissue sites in need of wound healing or protection from infection or other injurious agents. Prolonged immune response to the continued presence of such agents, or likewise the initiation of tumor development, which is known to resemble the process of wound healing, can result in chronic inflammation. The “wound-like” aspects of early tumor growth, where proteinases degrade the basal membrane surrounding tumor cells, allow cancerous cells to come in contact with stroma. Normal wound healing involves such stromal–epithelial interactions. However, multi-factor, inflammatory-mediated interaction between cancer cells and their microenvironment abnormally alters the composition of the stroma as the tumor develops, causing structural changes to the vasculature. This has been recognized as an opportunity for therapeutic intervention by targeting these effects on the tumor stroma, such as with angiogenesis inhibition therapies.

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THE KINETICS OF INFLAMMATION

The biology of acute edema, with peak neutrophilia and vascular leak occurring very early on, can best be imaged by a probe that clears quickly from the tissue, with a short tissue half-life and a peak signal at 1-3 hours after injection.

Once these peaks subside, neutrophils are replaced by a macrophage influx as vascular leak begins to resolve. This phase of inflammation, driven less by edema and more by cellular activity, can best be imaged using a probe with a long tissue half-life that will accumulate in the leaky areas and have a peak signal at 24 hours after injection.

Understanding the kinetics of inflammation and aligning with the characteristic blood and tissue half-life of the probe, as well as the timing of the injection, are important considerations for proper imaging of inflammation.

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PROBE HALF-LIFE & INJECTION TIMING

PROBE*	AGENT TYPE	BLOOD HALF-LIFE	TISSUE HALF-LIFE	OPTIMAL IMAGING TIME AFTER INJECTION	ROUTE OF METABOLISM/ BACKGROUND TISSUE(S)
IVISense Vascular 680 IVISense Vascular 750	PEGylated large scaffold	7 h	72 h	24 h (Imaging of vasculature for up to 4 h; tumor accumulation at 24 h)	Low liver lung
IVISense Vascular NP 680 IVISense Vascular NP 750	Nanoparticle	20 h	>100 h	24 h	Long term tissue accumulation
IVISense Edema 680	Albumin-binding small molecule	1.5 h	5 h	3 h (1-3)	Bladder
IVISense Tomato Lectin 680	Fluorescently labeled tomato lectin	2 h	>48 h	6 h	Overall vascular background

*Many other probes, such as IVISense Pan Cathepsin, IVISense MMP, IVISense Neutrophil Elastase, or IVISense Integrin Receptor, are capable of imaging aspects of inflammation by detecting biomarkers other than vascular leak. See the *Fluorescent Agents Reference Table* in the References section for a full list of probes and their specificity, routes of metabolism, and more.

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ENDOTHELIUM

Endothelium is comprised of a single layer of endothelial cells and forms the lining of blood vessels throughout the body. Angiogenic vessels that sprout to support tumor growth will often develop abnormally, with leaks in the endothelial layer due to oncogenic mutations which impair the proteins that join the endothelial cells together. This increased vascular permeability allows fluid to leak out and pool in surrounding tissues.

Many fluorescent vascular imaging agents have no specificity and work by passively circulating in the blood, such as IVISense Vascular NIR fluorescent probe, which can image vascularity and vascular leak. However, agents can also be designed to image vasculature by targeting the endothelium, binding the glycoproteins expressed on the luminal surfaces.

Tomato *(Lycopersicon esculentum)* lectin, a single polypeptide glycoprotein, can be conjugated to a fluor and validated to bind the sugar-containing proteins present on the surface of endothelial cells. IVISense Tomato Lectin 680 NIR fluorescent probe preferentially labels endothelial cells and can be used to non-invasively assess tumor vasculature in response to an anti-angiogenic drug, or to assess vascular burden as another biological consideration.

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TUMOR BURDEN
- Folate

METABOLIC ACTIVITY
- MMP

ANGIOGENESIS
- Cathepsin
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- Phosphatidylserine
- Endothelium
- αvβ3 Integrin

METASTASIS

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- Bombesin

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- Elastase
- Hydroxyapatite

CELL DEATH
- Transferrin

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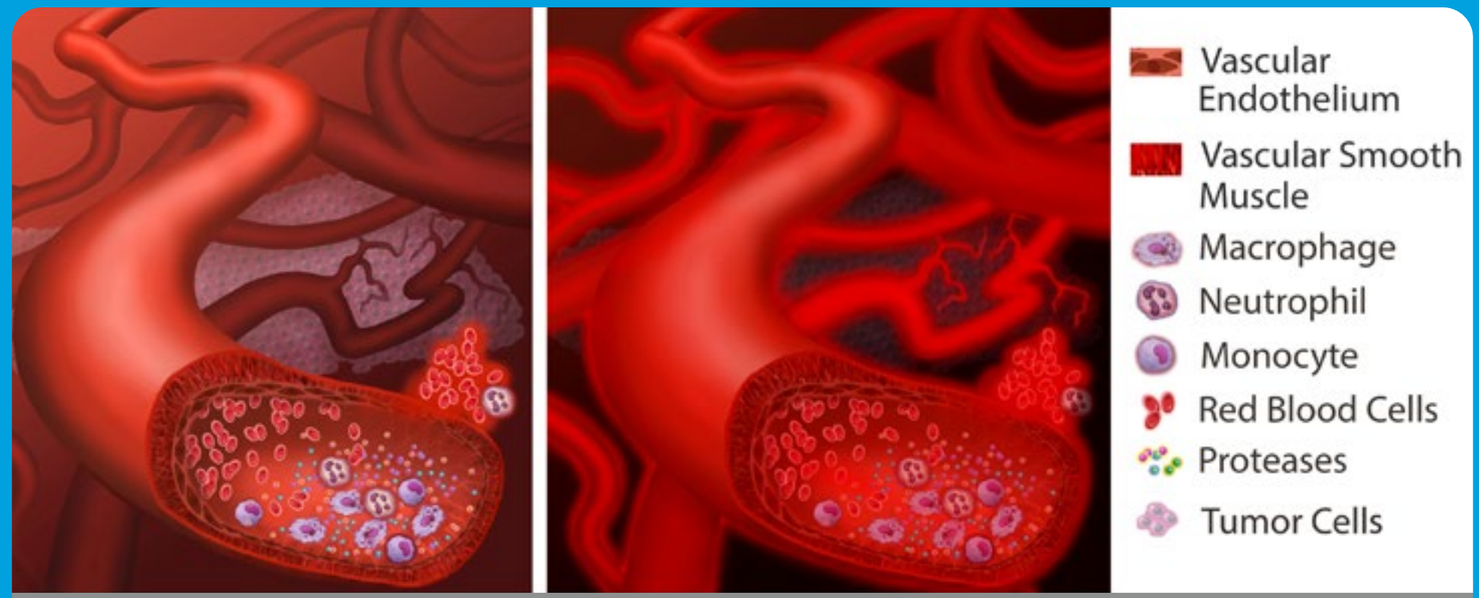
ENDOTHELIUM



Endothelial cells line the inside of blood vessels and can be targeted using fluorescent probes to image vasculature and tumor-related angiogenesis. IVISense Tomato Lectin fluorescent activation is represented in the figure on the right.

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LONGITUDINAL IMAGING OF FUNCTIONAL CHANGES IN TUMOR MICROVASCULATURE

Antiangiogenic agents have become a promising therapeutic pursuit in cancer therapy. Early in the development of these agents, it was thought that anti-angiogenesis strategies worked by blocking tumor angiogenesis, and that this effect could then limit the effectiveness of the agent itself, as well as other adjunct therapies - a paradoxical remodeling of tumor vasculature.[1,2] However, data has since demonstrated that anti-angiogenic drug treatments normalize vasculature, allowing for improved delivery of drugs to the tumor.[2]

This selective transient pruning of poorly formed vessels leads to a temporary improvement of blood flow and oxygen delivery[1], with vessel structure that yields less vascular leak around the tumor over the course of treatment. Additionally, vascular changes can result in improved delivery of chemotherapy and a corresponding increase in efficacy. These phenomena may be functionally demonstrated using fluorescent vascular imaging agents, providing powerful biological characterization of tumor biology and the effects of treatment, including a normalized vascular bed and tumor regression.

1. Rajabi M, Mousa SA. The Role of Angiogenesis in Cancer Treatment. *Biomedicines*. 2017 Jun 21;5(2):34.
2. Ackermann et al. Adnectin CT-322 inhibits tumor growth and affects microvascular architecture and function in Colo205 tumor xenografts. *Int J Oncol*. 2011 Jan;38(1):71-80.

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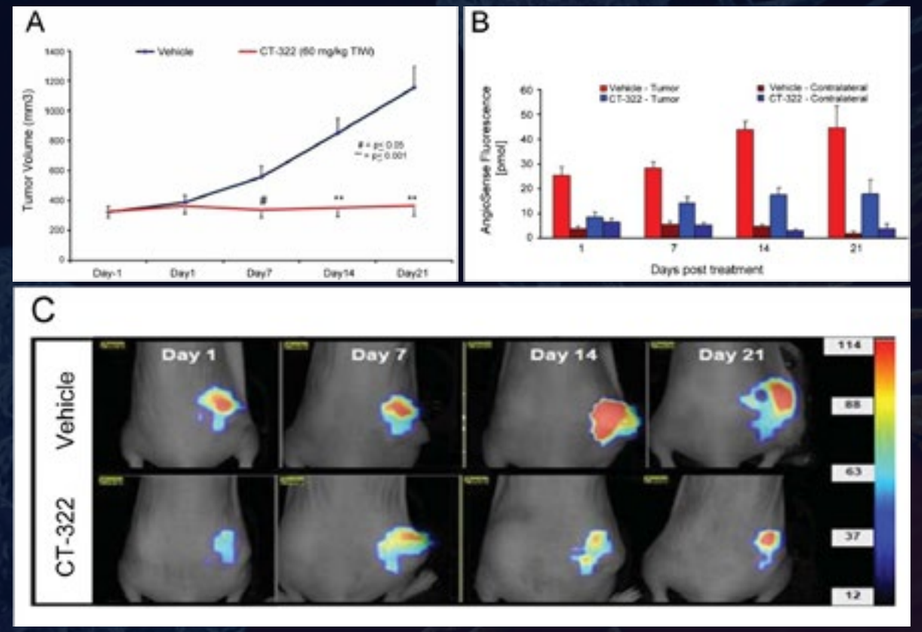
CASE STUDY: REDUCED ACCUMULATION OF VASCULAR IMAGING PROBE AFTER A SINGLE DOSE OF ANTIANGIOGENIC DRUG SUGGESTS REDUCED TUMOR VASCULAR PERMEABILITY

In this study by Ackerman, et al., the researchers used IVISense Vascular 680 (AngioSense 680) fluorescent probe and fluorescence tomography, to establish that the maximal anti-vascular effect of a potent antiangiogenic drug, CT-322, occurred within 24 h after the first drug administration, and this response mechanistically preceded the inhibition of tumor progression. The measurement of functional vascular leakiness with a vascular probe provided data that could not be easily achieved with histology or vascular corrosion casting.



IMAGED USING FLUORESCENCE MOLECULAR TOMOGRAPHY

A: Growth curves of Colo205 tumors on nude mice treated with vehicle (n=12) or CT-322 (n=12).

B: Tumor vascularity, AngioSense fluorescence quantitation (pmol) on days 1, 7, 14 and 21. Note the increase in fluorescence on days 7-21 in the vehicles that is paralleled by increases in tumor size. Non-tumor associated fluorescence was assessed in adjacent background flank areas within each mouse scan (contralateral).

C: Tumor Vascularity, IVISense Vascular (AngioSense) fluorescence images acquired 24 h post injection. Representative controls vs. CT-322 treated mice.

Ackermann et al. Adnectin CT-322 inhibits tumor growth and affects microvascular architecture and function in Colo205 tumor xenografts. *Int J Oncol*. 2011 Jan;38(1):71-80.

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BY CHARACTERIZATION | BY APPLICATION | BIOPROFILING

IVISense Probe	MW (g/mol)	Specificity	*In Vivo* Biology Use	Mouse Dose (25 g)	Sold As (Unit Size)	Optimal Imaging Time (h)
⭘ Vascular 680	~70,000	Passive; non-specific systemic circulation	Imaging of vascularity, perfusion, and vascular permeability.	2 nmol	24 nmol	24 h (Imaging of vasculature for up to 4 h; tumor accumulation at 24 h)
⭘ Vascular 750						
✶ Vascular NP 680	~4,000,000 (20-50 nm diameter)	Passive; non-specific systemic circulation	Imaging of vascularity, perfusion, and vascular permeability. Long pharmacokinetic profile.	100 uL	100 nmol (0.5 mL)	24 h
✶ Vascular NP 750						
⭘ Edema 680	~1,540	Passive; non-specific systemic circulation	Imaging of vascularity, perfusion, and vascular permeability. Short pharmacokinetic profile.	4 nmol	40 nmol	3 h (1-3)
⌖ Tomato Lectin 680	~72,000	Endothelial glycoproteins	Vascular burden assessment through binding of glycoprotein N-actylglucosamines on the surface of vascular endothelial cells.	2 nmol	24 nmol	6 h

⭘ Vascular and Physiological Probes are a range of highly fluorescent *in vivo* imaging molecules that remain highly stable and localized in the anatomy for various periods of time to enable imaging of disease physiology, vasculature, vascular permeability and angiogenesis.

✶ Highly fluorescent near infrared nanoparticles specifically designed for *in vivo* imaging. They remain localized in the vasculature for extended periods and enable imaging of blood vessels and angiogenesis.

⌖ Targeted Probes actively target and bind to distinct biomarkers, with highly specific targeting to key biological mechanisms.

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BY CHARACTERIZATION | BY APPLICATION | BIOPROFILING

Each vascular probe can detect vascularity in cancer and inflammation, and may be especially suitable for:

Tumor Imaging - Orthotopic mouse breast cancer
IVISense Vascular probes provide superior signal to background.

Acute Edema Imaging - Mouse model of acute, inflammatory paw edema
IVISense Edema 680 provides superior imaging capability.

Intravital Microscopy - Assessment of vessel morphometry
IVISense Vascular NP fluorescent nanoparticles are designed as highly robust agents for this application; Vascular NP shows the ability to image both tumors and inflammation with long term accumulation at the imaging site (can be a benefit for time course studies).

IVISense Probe	Edema 680	Vascular 680 Vascular 750	Vascular NP 680 Vascular NP 750	Tomato Lectin 680
Biomarker specificity	**Passive; non-specific systemic circulation**	**Passive; non-specific systemic circulation**	**Passive; non-specific systemic circulation**	Endothelial glycoproteins
Tumor Imaging	+	**+++**	++	++
Tumor Imaging Time	3 h	24 - 48 h	24 - 48 h	6-24 h (stable label of vasculature)
Tumor Washout	48 h	144 h	>192 h	>192 h
Response To Anti-Angiogenic Agents	*nd*	+++	*nd*	*nd*
Acute Edema Imaging	**+++**	+	+	-
Tissue Imaging Time	3 h	24 h	24 h	NA
Tissue Washout	48 h	144 h	>192 h	NA
Intravital Microscopy	++	**+++**	**+++**	**+++**
Optimal Imaging Time	5 – 15 min	5 – 60 min	5 min – 3 h	6-24 h

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BY CHARACTERIZATION | BY APPLICATION | BIOPROFILING

PerkinElmer offers a fluorescent Vascular Panel consisting of a carefully curated collection of probes to detect multiple aspects of vascular disease. Including probes at various wavelengths (680 nm and 750 nm), the panel offers the opportunity for multiplex imaging of appropriate probe combinations to maximize information gained from your research studies.

Vascular Panel

Set of Fluorescent Probes Bundled by Application

IVISense Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25g)	Amount (Unit Size)	Optimal Imaging Time (h)	Probe Clearance
Vascular 680	~70,000	Vascularity	Leaks into sites of edema	2 nmol	24 nmol	24 h	6-7 d
MMP 750 FAST	43,000	Matrix Metalloprotease family proteases	Cleaved by multiple metalloproteinases - secreted markers of neutrophils, macrophages, and mast cells	2 nmol	24 nmol	24 h (12-24)	6-7 d
Integrin Receptor 680	1,432	αVβ3 integrin	Detection of integrin αvβ3 expression in tumors and neovasculature.	2 nmol	24 nmol	24 h	14 d
Cat B 680 FAST	33,000	Cathepsin B	Selectively cleaved by cathepsin B proteinases upregulated in tumors and inflammatory cells.	2 nmol	24 nmol	6-24 h	3 d
Pan Cathepsin 750 FAST	22,500	Cathepsin family proteases	Versatile pan-cathepsin-activatable agent	4 nmol	48 nmol	6-24 h	6-7 d

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THE HALLMARKS OF METASTASIS[1]

The leading cause of morbidity and mortality in cancer patients is metastasis; when tumor cells spread from a primary tumor and establish secondary tumors at distant sites in the body. Tumors form when subpopulations of cells with genomic instability undergo morphological and biochemical changes, becoming neoplastic. The cells may maintain their neoplastic state, proliferating abnormally while never progressing to malignancy. The complex process leading to malignancy and metastasis begins long before a primary tumor is detectable. Some of the neoplastic cells acquire additional traits, including aberrant active cytoskeletal rearrangement, that give them the ability to move around neighboring cells. Upregulation of a variety of proteases can allow tumor cells to degrade extracellular matrix, disrupt the basement membrane, and penetrate underlying stroma. This sets the stage for possible dissemination and growth into other tissues.

Although invasion is intrinsic to metastasis, the ability to penetrate the stroma by itself is not nearly sufficient to ensure metastasis will occur. Some tumors remain only locally invasive, never spreading to a secondary site. In other instances, as many as 4 x 10^6 cells per gram of tumor can disseminate per day, yet the overwhelming majority of these cells fail to colonize. Though metastatic potential exists on a spectrum and is not binary, none the less if an invasive cell cannot complete any of the subsequent steps in the metastatic cascade, it will not form a metastasis. To successfully metastasize, in addition to motility and invasion, a cell must further acquire the abilities to modulate the secondary site or local microenvironments, maintain plasticity as needed, and colonize secondary tissues, proposed by Welch and Hurst as the four hallmarks of metastasis.

1. Welch , Danny R. and Hurst, Douglas R. Defining the Hallmarks of Metastasis. *Cancer Res* June 15, 2019 (79) (12) 3011-3027.

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DETECTING METASTASES

Tumors must generally reach a certain size (~100-1000 mm^3) before becoming biologically and physiologically active at a detectable level. Though it presents an imaging challenge, detection of small metastatic tumors can be done and is best accomplished by choosing an imaging probe that aligns with the operant biology within the tumor. For example, the biological complexity of large tumors/metastases will include angiogenesis, inflammation, hypoxia, inflammation, and necrosis as well as upregulation of a number of additional biomarkers. In contrast, very small metastases may show very few of these biological abnormalities, so probes for specific tumor markers or early biological abnormalities may be most effective.

Imaging probes should be designed and validated to possess characteristics (clearance rate and route of metabolism) biologically appropriate for tumor/metastasis imaging and specific for relevant biomarkers to yield the best results. As the tumor grows, it becomes easier to detect, due to size and biological complexity, increasing the range of possible imaging probes to quantify such biological changes in real time.

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CLEARANCE RATES AND ROUTES OF METABOLISM

IVISENSE PROBE*	BIOMARKER SPECIFICITY	BLOOD HALF-LIFE	TISSUE HALF-LIFE	ROUTE OF METABOLISM / BACKGROUND TISSUES
Cat B 680 FAST Cat B 750 FAST	Cathepsin B	1 hr	36 hr	Salivary Glands > Liver, Kidneys
Cat K 680 FAST	Cathepsin K	30 min	36 hr	Kidneys > Liver
Pan Cathepsin 750 Pan Cathepsin 750 FAST	Cathepsin proteases	5 hr 1 hr	72 hr 36 hr	Low liver, intestine Low liver, bladder
MMP 645 FAST MMP 680 MMP 750 FAST	Matrix Metalloprotease	5 hr	72 hr	Liver > Kidneys Liver Liver > Kidneys
Integrin Receptor 645 Integrin Receptor 680 Integrin Receptor 750	αVβ3 Integrin	10 min 10 min 30 min	48 hr 24 hr 24 hr	Bladder, Kidneys Kidneys, Intestines Kidneys
Bombesin Receptor 680	Bombesin receptor	1.5 hr	96 hr	Pancreas, Kidney
Osteo 680 Osteo 750 Osteo 800	Hydroxyapatite	5-10 min 5-10 min 5-10 min	~30 days 7-10 days 7-10 days	Bladder

*IVISense Pan Cathepsin 680 is not included in this table because it is not recommended for imaging small tumors due to higher background signal in the liver.

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MMP

CATHEPSIN | αvβ3 INTEGRIN | BOMBESIN | HYDROXYAPATITE

Matrix metalloproteases (MMPs) are calcium-dependent, zinc-containing endopeptidases that play a variety of roles in tissue remodeling by degrading the extracellular matrix. Normally under strict regulation, controlled levels of matrix degradation are necessary to maintain healthy biological tissues. However, when cell signaling pathways are co-opted by tumor cells, abnormal activation of MMPs can restructure tissues in ways that facilitate cellular dispersal and tumor cell invasion. Tending to localize in tumor margins, MMPs play an important role in tumor metastasis and correlate with advanced cancer.

The ability to detect active MMPs upregulated and secreted at the site of metastasis can reveal distinctive local biology as well as assess therapeutic efficacy of specific drugs, sometimes prior to overt biological changes. MMPSense® NIR fluorescent imaging agents are able to detect a broad range of active MMPs, including MMP 2, 3, 7, 9, 12, and 13, to track tumor progression or evaluate the potential therapeutic efficacy of drugs targeting the underlying mechanisms involved.

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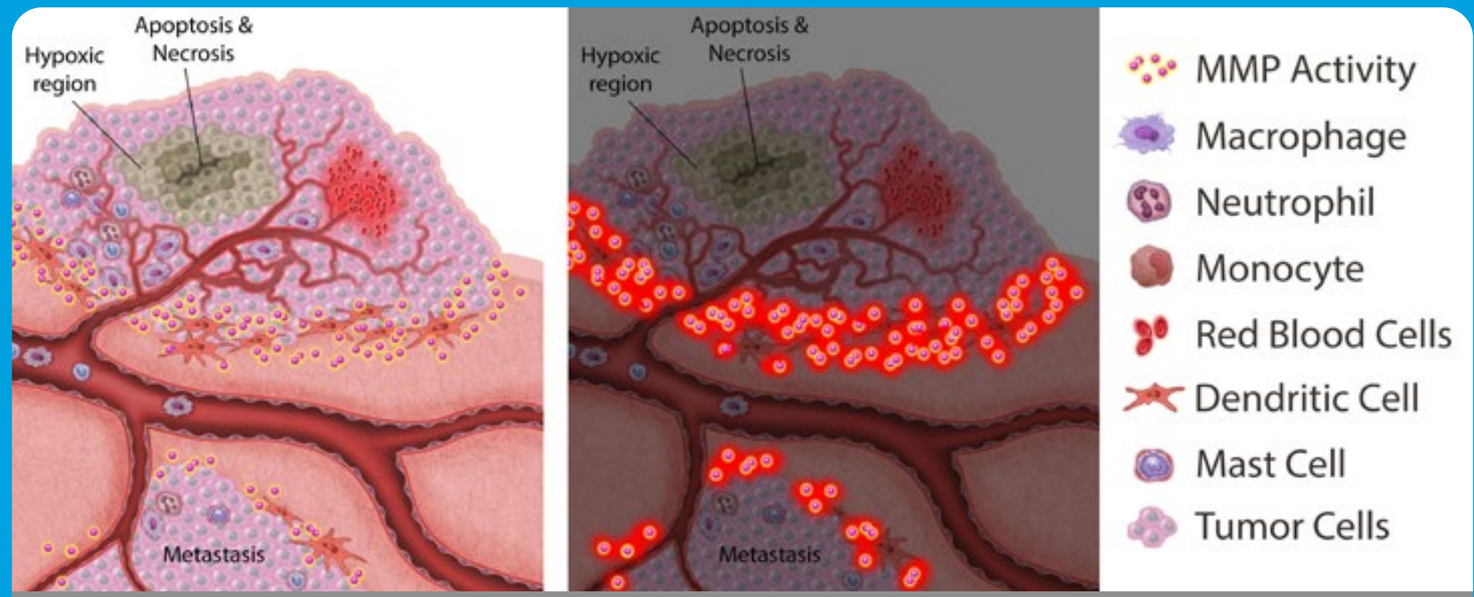
MMP | CATHEPSIN | αvβ3 INTEGRIN | BOMBESIN | HYDROXYAPATITE



Matrix metalloproteases are active in regions of tissue remodeling, in this case highly expressed and localized at the invasive tumor margins. IVISSense MMP fluorescent activation is represented in the figure on the right.

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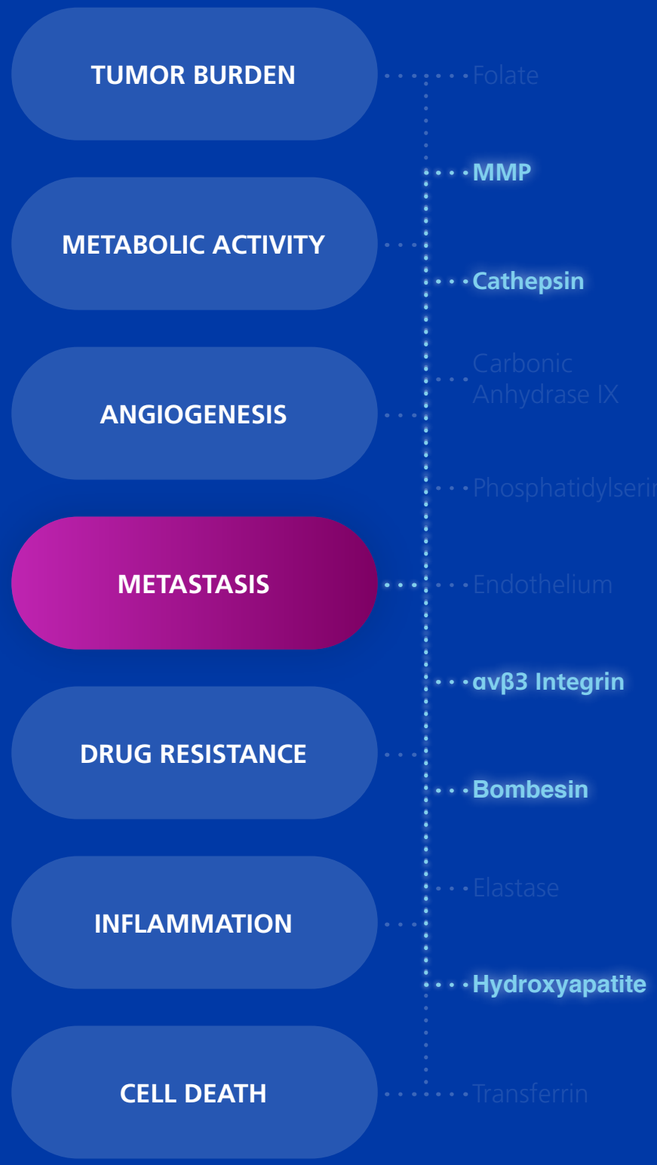

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MMP | **CATHEPSIN** | αvβ3 INTEGRIN | BOMBESIN | HYDROXYAPATITE

Associated with tumor aggression, cathepsins are a family of mostly lysosomal proteases that are pivotal components of signaling pathways which can enhance cancer cell growth and inflammation and contribute to matrix degradation, thereby facilitating tumor cell invasion.

Cathepsin B: Correlates with invasive and metastatic capabilities of many tumors, such as breast cancer where high expression levels have been linked to highly aggressive tumors; also upregulated in a variety of inflammatory cells (including eosinophils, neutrophils, and macrophages) capable of exerting either anti- or pro-cancer effects and contributing to motility and cellular invasion.

Cathepsin K: A lysosomal cysteine protease expressed in osteoclasts, chondrocytes, and synovial fibroblasts; involved in bone resorption and collagen degradation; high levels of expression are a useful marker for breast cancer and bone metastases.

IVISense Pan Cathepsin fluorescent imaging probes: versatile pan-cathepsin-activated agents.
IVISense Cat B fluorescent imaging probes: selectively cleaved by cathepsin B proteinases.
IVISense Cat K fluorescent imaging probes: selectively cleaved by cathepsin K proteinases.

For specific detection of tumors with associated cathepsin activity, NIR fluorescent imaging probes IVISense Pan Cathepsin, Cat B, and Cat K enable non-invasive longitudinal imaging of tumor progression via protease expression.

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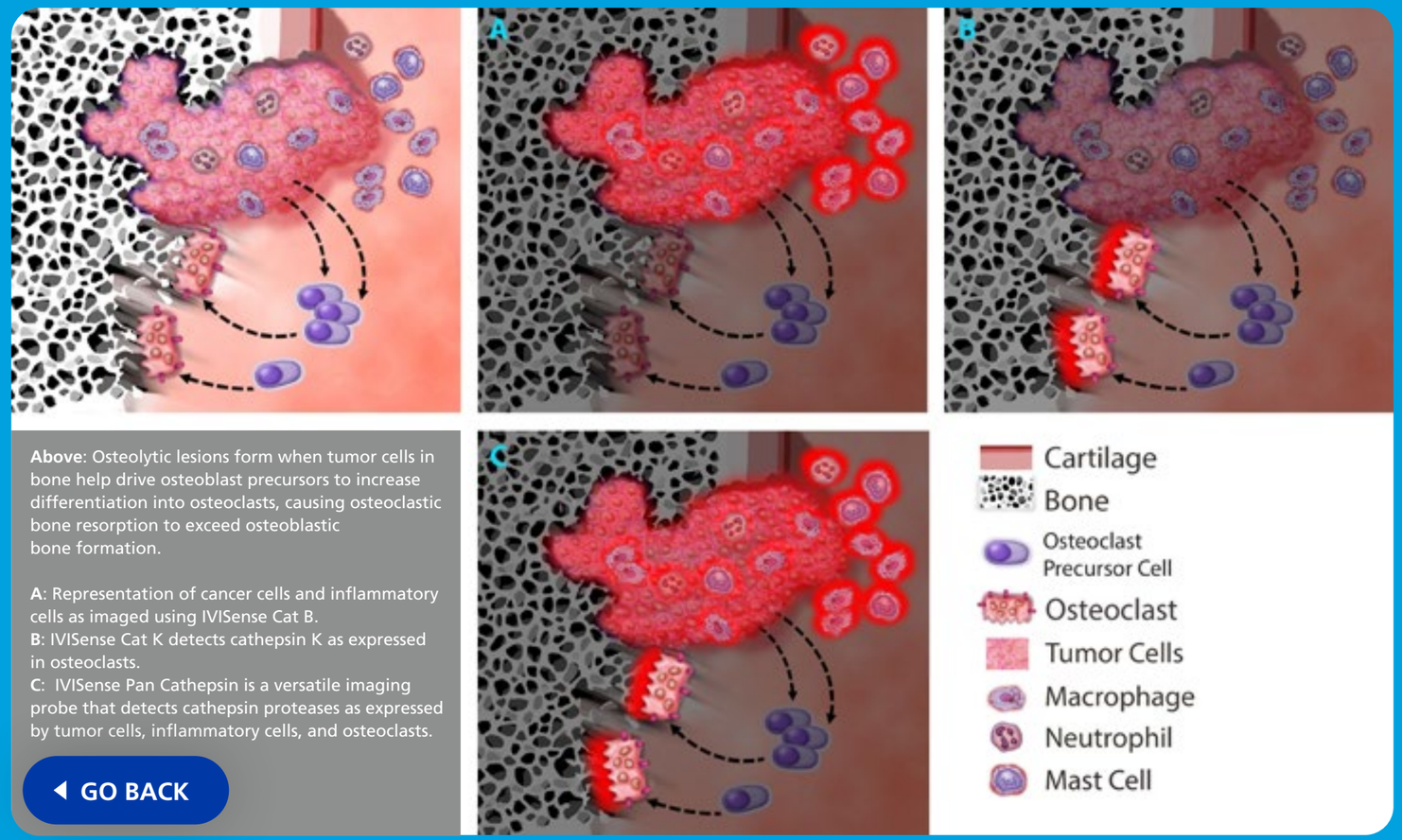

Above: Osteolytic lesions form when tumor cells in bone help drive osteoblast precursors to increase differentiation into osteoclasts, causing osteoclastic bone resorption to exceed osteoblastic bone formation.

A: Representation of cancer cells and inflammatory cells as imaged using IVISense Cat B.
B: IVISense Cat K detects cathepsin K as expressed in osteoclasts.
C: IVISense Pan Cathepsin is a versatile imaging probe that detects cathepsin proteases as expressed by tumor cells, inflammatory cells, and osteoclasts.

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MMP | CATHEPSIN | αvβ3 INTEGRIN | BOMBESIN | HYDROXYAPATITE

Integrins are cell surface signaling molecules that are essential for regulating communication between cells and their microenvironment. Key to maintaining the integrity of healthy tissue, integrins are critically involved in the cells' ability to adhere to the extracellular matrix. Likewise, they are key components in cellular migration, and thus are implicated in nearly every step of cancer progression including metastasis. Altered integrin expression can support cancer cell migration and invasion, playing an important role in metastatic colonization and facilitating survival of anchorage-independent circulating tumor cells.[1]

In particular, αvβ3 integrin is significantly upregulated in activated endothelial cells during neoangiogenesis, required early on for colonized cells to establish a secondary location. As a distinct biomarker of tumor progression, αvβ3 integrin expression can be imaged using IVISense Integrin Receptor, an NIR fluorescent imaging probe. IVISense Integrin Receptor probe provides excellent tumor definition with minimal off-target distribution due to its high affinity (Kd = 4.2 nM) and selectivity 5-20x over RDG peptide-based agents. With improved circulation half-life and specificity, it enables detection and measurement of αvβ3 integrins *in vivo*.

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1. Hamidi, H., Ivaska, J. Every step of the way: integrins in cancer progression and metastasis. *Nat Rev Cancer* **18**, 533–548 (2018).

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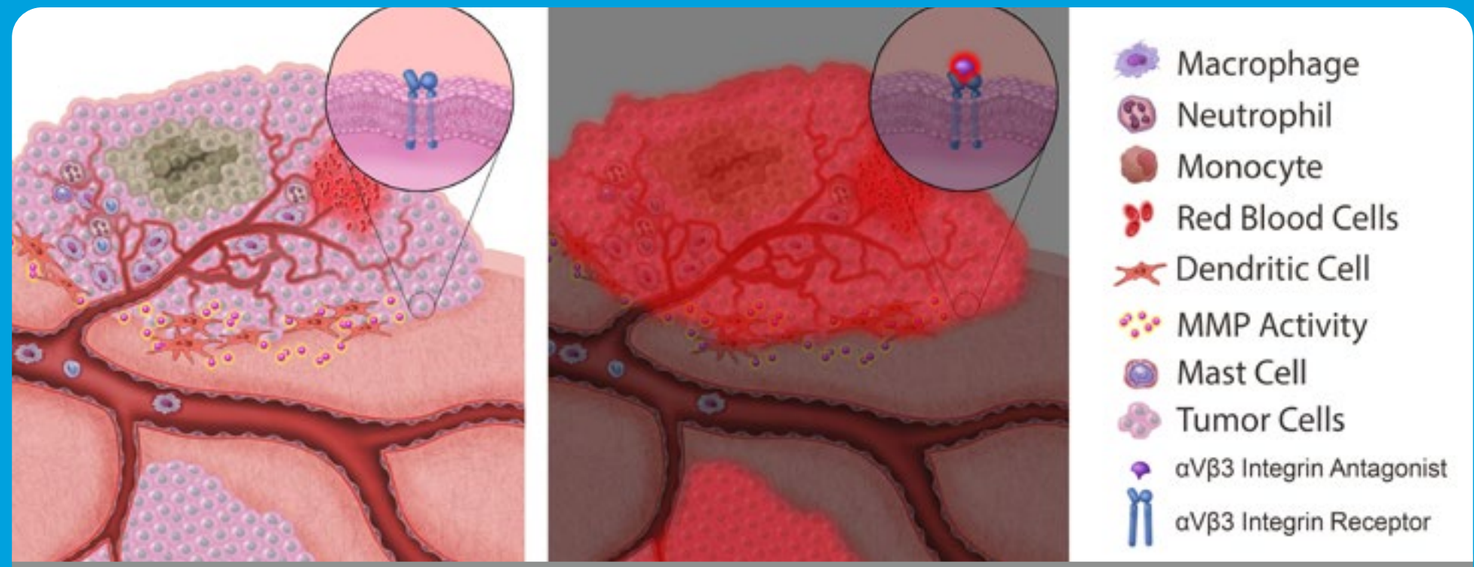

αvβ3 integrin is significantly upregulated in tumor cells and correlates with tumor aggressiveness. IVISense Integrin Receptor fluorescent imaging of fine tumor definition is represented in the figure on the right.

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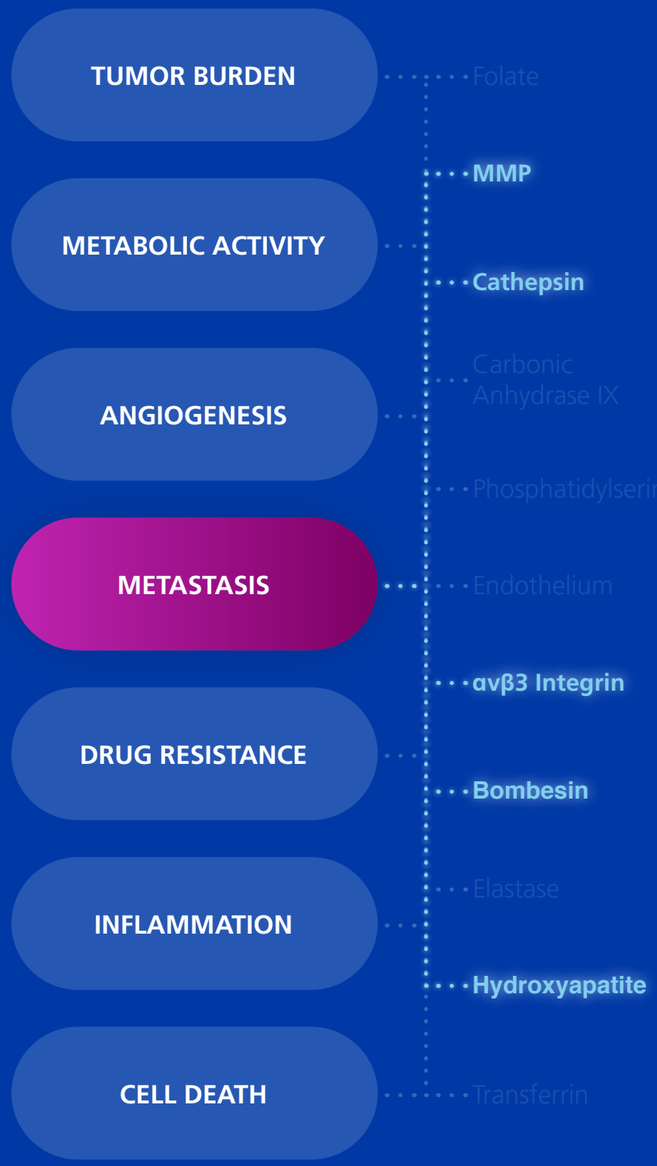

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MMP | CATHEPSIN | αvβ3 INTEGRIN | **BOMBESIN** | HYDROXYAPATITE

Bombesin is a 14-amino acid peptide originally isolated from the European fire-belly toad (*Bombina bombina*). Bombesin-like peptides and bombesin receptors (BBRs) have been the subject of investigation for the past two decades because of their involvement in cancer cell energy metabolism and proliferation. Mammalian bombesin analogs, such as gastrin-releasing peptide (GRP), can promote cell growth, malignant transformation, and tumor differentiation, such that many types of human cancer, including prostate, breast, lung, CNS, gastric, colon, and renal, show upregulated expression of BBRs on the surface of tumor cells.[1]

As potential targets for drug delivery, further exploration of these receptors is warranted. IVISense Bombesin Receptor, a novel NIR fluorescent imaging probe, is comprised of a 7-amino acid bombesin peptide analog, an NIR fluorophore, and a pharmacokinetic modifier to improve its plasma availability. This is an ideal probe to target and quantify upregulation of bombesin receptors *in vivo*, associated with tumor proliferation and metabolic activity, and this marker downregulates rapidly under anti-metabolic treatment regimen, like is seen in 18F-FDG PET imaging.[1]

VIEW IMAGE ▶

1. Tseng et al. Fluorescence imaging of bombesin and transferrin receptor expression is comparable to 18F-FDG PET in early detection of sorafenib-induced changes in tumor metabolism. *PLoS ONE* 12(8): 2017. e0182689.

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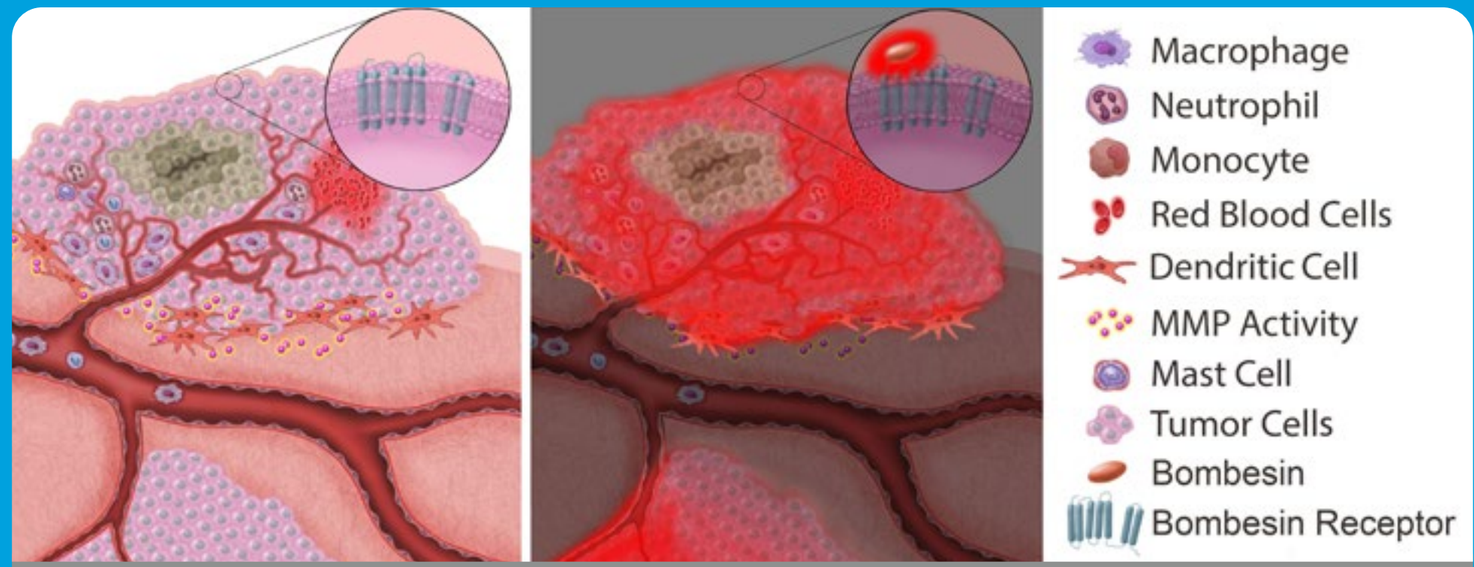

Depending on the tissue of origin, tumors overexpress metabolic receptors that can be used as biomarkers of cancer progression. The image above illustrates surface membrane receptors for bombesin. The panel on the right illustrates fluorescence for the bombesin receptor, showing less activity in dead, hypoxic, or poorly vascularized regions, where metabolic activity is reduced.

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- Endothelium
- αvβ3 Integrin

DRUG RESISTANCE
- Bombesin

INFLAMMATION
- Elastase
- Hydroxyapatite

CELL DEATH
- Transferrin

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The inorganic component of bone is mainly comprised of hydroxyapatite (HA), the major mineral product of osteoblasts for new bone formation. HA levels can serve as a useful biomarker for osteoblast activity, where abnormal exposure of HA can indicate a disturbance to bone homeostasis, such as in bone metastasis.

Bone is one of the most frequent sites for tumor metastasis and is the prevailing site for breast and prostate cancer metastasis. The metastatic site is not random; dispersed cells can only grow in environments that are favorable for colonization. As such, the bone microenvironment appears to provide an especially conducive niche that enables the somewhat distinct process of metastasis as it occurs in bone, from what can be a very long dormancy to reactivation to cellular proliferation.

IVISense Osteo NIR fluorescent bisphosphonate imaging probes bind with high affinity to HA both *in vitro* and *in vivo*, using subtherapeutic doses for imaging. IVISense Osteo fluorescent probes have been shown to bind to active osteoblastic and osteoclastic surfaces of bone[1], enabling *in vivo* detection, measurement, and monitoring of skeletal changes and progression of bone metastasis.

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1. Kozloff, K.M., Weissleder, R. and Mahmood, U. (2007), Noninvasive Optical Detection of Bone Mineral. *J Bone Miner Res*, 22: 1208-1216.

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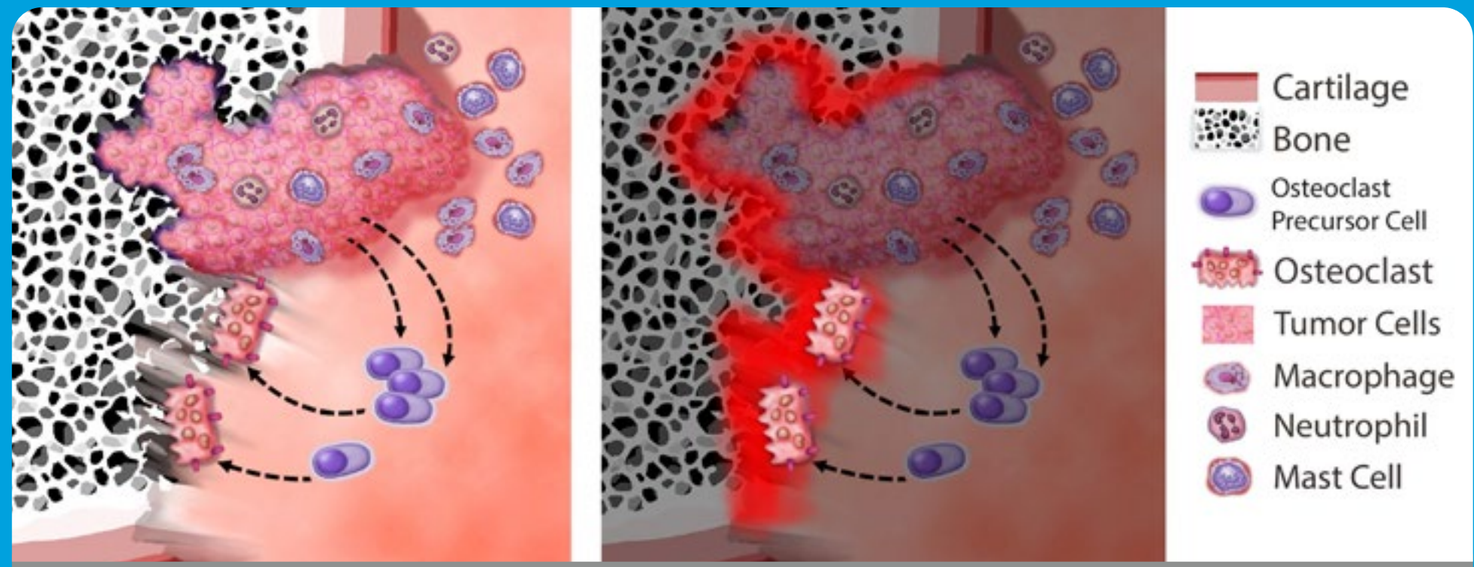

Osteolytic lesions form when tumor cells in bone help drive osteoblast precursors to increase differentiation into multinucleated osteoclasts, causing osteoclastic bone resorption to exceed osteoblastic bone formation.

Hydroxyapatite (HA) is exposed during bone resorption and can be a biomarker of osteoclast activity. IVISense Osteo binds to HA, enabling *in vivo* detection of skeletal remodeling as shown in the image on the right.

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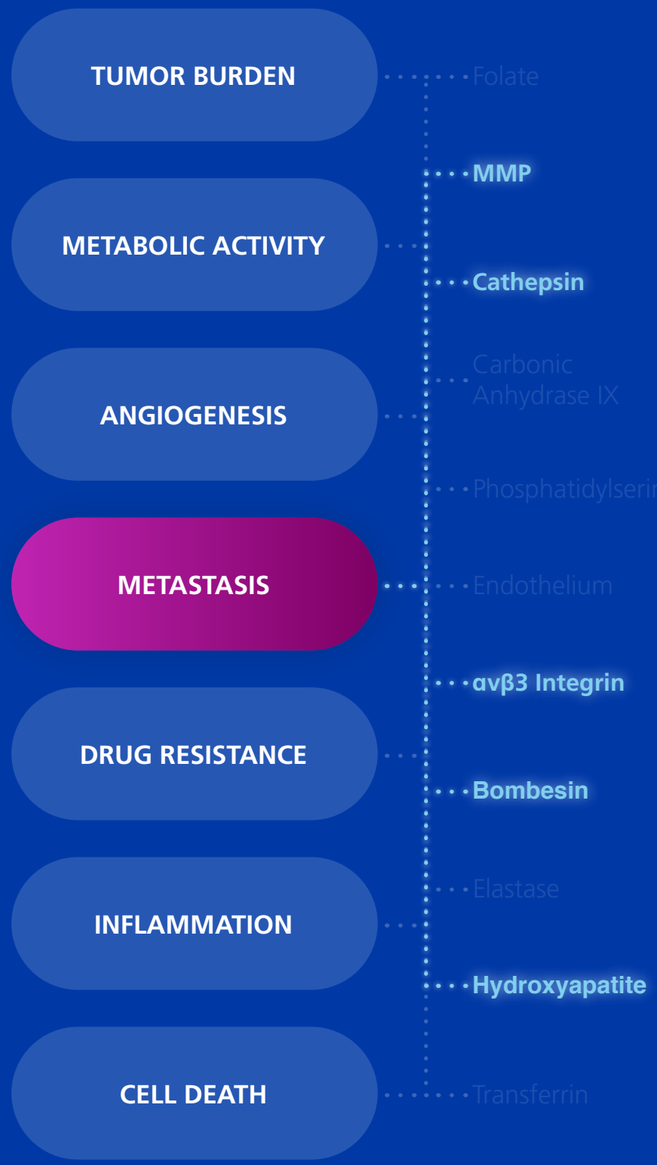

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QUANTIFYING AND COMPARING COMPLIMENTARY BIOMARKERS AS INDICATORS OF TREATMENT RESPONSE

Healthy bone is maintained by a strict balance of continuous bone formation and resorption. When this dynamic equilibrium is upset, an increase or decrease in activity of one cellular player may be better understood by also characterizing changes in its functional counterpart.

Bone cells (i.e. osteoblasts, producing hydroxyapatite for new bone formation, and osteoclasts, secreting cysteine proteases, including cathepsin K, for bone resorption) are influenced by cancer cells in mainly two ways[1]: most commonly, tumor cells in bone will drive osteoblast precursors to increase differentiation into multinucleated osteoclasts while also inhibiting osteoblasts. The resulting prevalence of osteoclastic bone resorption over osteoblastic bone formation leads to osteolytic lesions.

A less common occurrence is when cancer cells stimulate osteoblast precursors to increase differentiation into osteoblasts, tipping the balance toward more new bone deposition, or osteoblastic lesions. These bulging growths have abnormal architecture which weakens the structural integrity of the bone despite its increased mass. Although osteolytic lesions are more commonly seen than osteoblastic lesions, neither is exclusive and often will occur together in the same individual with bone metastasis.[1]

Fluorescence molecular imaging of bone cancer can detect changes in the rates of bone resorption and formation *in vivo* for a more complete characterization of disease progression or therapeutic effectiveness. The ability of a drug to block the influence of cancer cells by either increasing bone formation or slowing bone resorption (in the case of osteolytic lesions) can be tracked by quantifying and comparing changes in the levels of exposed hydroxyapatite (HA) to changes in cathepsin K (cat K) expression. When tracked together in the same animal, the effects of a drug can be more clearly defined with a deeper understanding of the mechanisms at play.

1. Ottewell PD. The role of osteoblasts in bone metastasis. *J Bone Oncol*. 2016;5(3):124-127.

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CASE STUDY: MULTIMODALITY IMAGING OF TUMOR AND BONE RESPONSE IN A MOUSE MODEL OF BONY METASTASIS[1]

Recent research focusing on targeted agents that disrupt specific intricately-involved signaling pathways in cancer presents both possibilities and challenges. The highly complex effects of these treatments involve numerous mechanisms, making them difficult to characterize and sometimes eliciting a treatment response with no drastic morphologic changes. In this case study, the authors explored imaging methods that possess the sensitivity and specificity needed to measure the affects of these targeted agents. By using fluorescence molecular imaging in conjunction with other imaging methods, the authors were able to quantify the response to two drugs in a mouse model of osteolytic bony metastasis; docetaxel, a chemotherapy drug used to kill cancer cells by halting cell division, and zoledronic acid (ZA), a bisphosphonate used to slow the effects of osteoclasts on bone.

Intratibial implantations of breast cancer cells (MDA-MB-231) were monitored using a multimodal approach that included functional assessment by fluorescence imaging. After treatment with either docetaxel and ZA, each animal was imaged with two probes. IVISense Osteo 800 fluorescent probe was used to image the amount of exposed hydroxyapatite (HA) resulting from both osteoblastic and osteoclastic activity, and IVISense Cat K 680 FAST was used to image cathepsin K expressed by osteoclasts.

The results showed significant bone remodeling changes at weeks 2 and 4 in the docetaxel group, and no significant change detected in the ZA or control group. For the IVISense Cat K signal, positive change indicated bone loss, while negative change indicated bone growth. The IVISense Osteo signal was interpreted to mean the inverse. The comparison of the IVISense Osteo signal to the Cat K signal provided the context for a very elegant approach to delineating drug effects.

Using multiple imaging modalities and cleverly pairing IVISense Cat K and IVISense Osteo probes to understand the complex interplay of osteolytic and osteoblastic activity, the authors were able to characterize changes occurring under different treatment regimens.

1. Hoff BA, Chughtai K, Jeon YH, et al. Multimodality imaging of tumor and bone response in a mouse model of bony metastasis. *Transl Oncol*. 2012;5(6):415-421.

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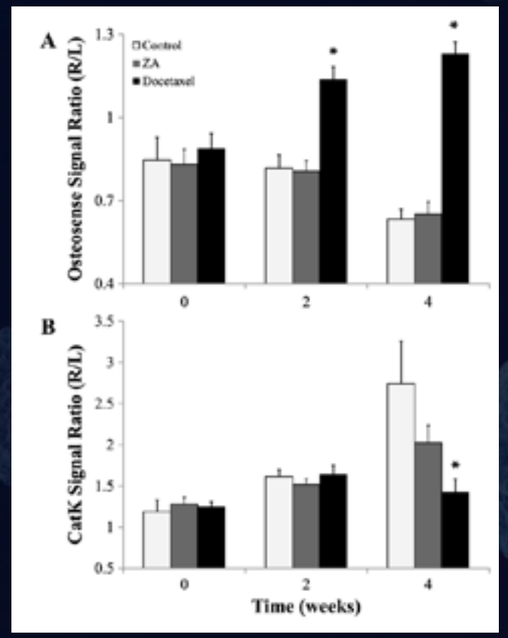

Figure 1: Bar plots of fluorescent signals in the tumor-bearing leg from (A) IVISense Osteo 800 (OsteoSense 800) and (B) IVISense Cat K 680 FAST are presented as values normalized by the non-tumor bearing leg. The ZA group showed no significant difference from controls with either fluorescent probe, but the docetaxel group showed significant increases in IVISense Osteo 800 uptake (A, black bar) on weeks 2 and 4 and a significant drop in IVISense Cat K 680 FAST signal (B, black bar) on week 4. Asterisk indicates a significant difference from the control group ($P < .05$).

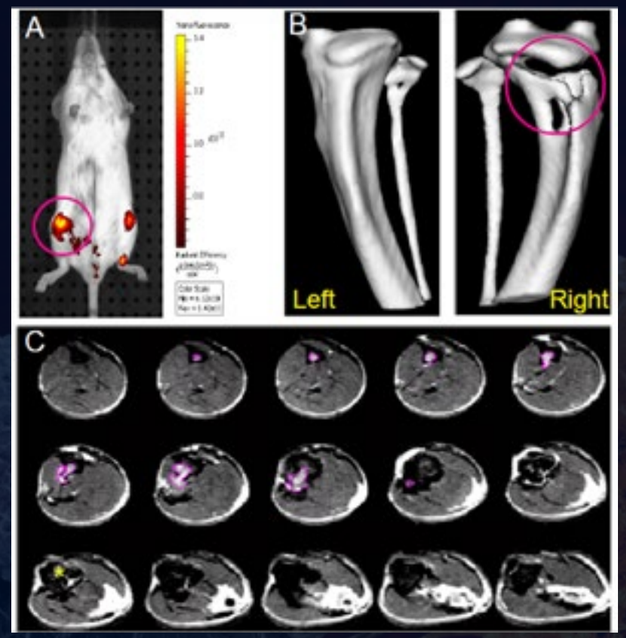

Figure 2: Assessment of osteoblastic activity, bone integrity, and tumor burden by multi-modality imaging. **(A)** Fluorescence image using OsteoSense 800 clearly identifies the right leg (magenta circle) as having increased osteoblastic activity relative to the left (control) leg. **(B)** The extend of bone damage as a result of the tumor is clearly observed in the microCT surface rendering of the right tibia (Right) with metastatic cancer. The healthy tibia (Left) shows no appreciable loss in bone density. **(C)** Finally, the location (magenta contour) and extent of tumor burden can be assessed by magnetic resonance imaging (MRI) of a tumor (~10 µL) in the right tibia. The MRI data is presented as individual slices going from the foot (top-left) to the mid-region of the femur (bottom-right). The location of the knee is indicated by the yellow asterisk. All of these images were acquired in the same animals approximately 25 days post-implantation. *Image courtesy of Craig Galban, PhD, University of Michigan.*

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BY CHARACTERIZATION | BY APPLICATION

IVISense Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25 g)	Sold As (Unit Size)	Optimal Imaging Time (h)
✂ Cat B 680 FAST	33,000	Cathepsin B	Selectively cleaved by cathepsin B proteinases upregulated in tumors and inflammatory cells	2 nmol	24 nmol	6-24 h
✂ Cat B 750 FAST	23,000			4 nmol	48 nmol	6-24 h
✂ Cat K 680 FAST	8,500	Cathepsin K	Selectively cleaved by cathepsin K proteinases upregulated in osteoclasts and tumor-associated macrophages.	2 nmol	24 nmol	6-24 h
✂ Pan Cathepsin 680	~400,000	Cathepsin proteases	Pan-cathepsin activatable agent that detects abnormal upregulation of cathepsin secretion associated with cancer and inflammation.	2 nmol	20 nmol	24 h (24-48)
✂ Pan Cathepsin 750	~450,000			2 nmol	24 nmol	24 h
✂ Pan Cathepsin 750 FAST	22,500			4 nmol	48 nmol	6-24 h
✂ MMP 645 FAST	43,000	Matrix Metalloprotease	Cleaved by multiple metalloproteinases associated with many disease areas including cancer and inflammation.	4 nmol	48 nmol	24 h (6-24)
✂ MMP 680	~450,000			2 nmol	20 nmol	24 h (24-36)
✂ MMP 750 FAST	43,000			2 nmol	24 nmol	24 h (12-24)
⌖ Integrin Receptor 645	1,250	αVβ3 Integrin	Detection of integrin αVβ3 expression in tumors and neovasculature.	2 nmol	24 nmol	6-24 h
⌖ Integrin Receptor 680	1,432			2 nmol	24 nmol	24 h
⌖ Integrin Receptor 750	1,278			2 nmol	24 nmol	24 h
⌖ Bombesin Receptor 680	24,000	Bombesin receptor	Detects upregulation of bombesin receptors on tumor cells associated with increased tumor proliferation.	2 nmol	24 nmol	24 h
⌖ Osteo 680	1,471	Hydroxyapatite	Binds exposed hydroxyapatite (HA), a biomarker for osteoblast activity; abnormal HA levels can indicate bone damage/disease, such as in metastasis.	2 nmol	24 nmol	3-24 h
⌖ Osteo 750	1,101			4 nmol	48 nmol	3-24 h
⌖ Osteo 800	1,281			2 nmol	24 nmol	3-24 h

✂ Activatable probes are optically silent upon injection and become highly fluorescent following protease-mediated activation. Activatable FAST agents are designed with a novel small molecule architecture that confers an accelerated pharmacokinetic profile with earlier imaging time points.

⌖ Targeted probes actively target and bind to distinct biomarkers with highly specific targeting to key biological mechanisms.

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BY CHARACTERIZATION

BY APPLICATION

IVISense Probe	Cat B	Cat K	Pan Cathepsin		MMP		Integrin Receptor (αVβ3)	Bombesin Receptor	Osteo (Hydroxyapatite)
Available Versions	680 FAST 750 FAST	680	680	750 FAST 750	680	645 FAST 750 FAST	645 680 750	680	680 750 800
Applications									
Early Tumor Assessment	+++	+	+++	+++	+++	+++	++++	+++	+
Metastasis Imaging	++	Bone resorption: ++++	+/- due to liver interference	++	+/- due to liver interference	++	++	+++	Bone growth or resorption: ++++
Tumor Microenvironment	-	+++	-	-	-	-	-	-	+++
Image Tumor Vascularity	-	-	-	-	-	-	+++	-	-
Assessing Treatment Efficacy	+++	+++	+++	+++	+++	+++	+++	++++	+++
Probe Characteristics									
Imaging Time Post Probe Injection	6-24 hr	24 hr (24-36)	24 hr (24-48)	6-24 hr 24 hr (24-48)	24 hr (24-36)	24 hr (6-24) 24 hr (12-24)	6-24 hr 24 hr 24 hr	24 hr	3-24 hr
Tumor Washout	3 days	6-7 days	6-7 days	3 days 6-7 days	6-7 days	6-7 days	6-7 days 14 days 4-6 days	6-7 days	Preimage subtraction

Summary of probes and applications. An overview of results, 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.

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Therapeutic resistance is a complex process that can arise from multiple origins. It occurs in all cancer types and in all therapy methods, including chemotherapy, immuno-therapy, and novel targeted drug therapy.[1] Broadly, tumor cells can acquire drug resistance as an adaptive response during treatment or it can be intrinsic to the cells, existing prior to treatment and manifesting in ways that allow some cells to survive and proliferate while others die.

Tumor microenvironment plays a critical role in the development of therapeutic resistance and in malignant progression. Most notably, tumor hypoxia and acidosis have been identified as conditions that profoundly influence cancer cell biology and lead to inhibition of the therapeutic response. Hypoxia is a pathological condition where tissue is deprived of adequate oxygen. In normal cells, hypoxia typically leads to cell death. However, in cancer cells, hypoxic conditions can directly or indirectly enable some cells to survive and proliferate. Hypoxia can induce epigenomic and genomic changes which, among other adaptations, can enable cells to survive the poor nutrient supply of the harsh tumor microenvironment by altering the tumor metabolism. This can function as a mechanism of drug resistance, allowing cells to evade drugs that preferentially target rapidly dividing cells. Under selective pressure from therapy, surviving cells can go on to form subpopulations of resistant cells that can lead to clinical relapse.

Acidosis of the tumor microenvironment is an important epigenomic change that occurs in response to hypoxia, and this is regulated by the expression of carbonic anhydrase IX (CA IX). Rarely expressed in normal human tissue, CA IX is highly over-expressed in many cancer types in response to low oxygen, which is driven by HIF-1α one of a family of transcription factors called hypoxia-inducible factors (HIFs).[2] Although this makes CA IX an excellent biomarker for hypoxia, the main role of CA IX is in promoting an acidic extracellular environment, and acidosis of the microenvironment that facilitates tumor cell growth, invasion, survival, and mechanisms of drug resistance. Research using CA IX inhibitors is helping to better define and establish the importance of CA IX in metastasis, poor prognosis, and resistance to therapeutic interventions, making it a highly relevant biomarker in the study of hypoxia and acidosis in cancer therapy.[3]

1. Wang X, Zhang H, Chen X. Drug resistance and combating drug resistance in cancer. *Cancer Drug Resist* 2019;2:141-160.
2. Al Tameemi Wafaa, Dale Tina P., Al-Jumaily Rakad M. Kh, Forsyth Nicholas R. Hypoxia-modified cancer cell metabolism. *Frontiers in Cell and Developmental Biology* 2019; 7:4.
3. Andreucci E, et al. The carbonic anhydrase inhibitor SLC-0111 sensitises cancer cells to conventional chemotherapy. *J Enzyme Inhib Med Chem* 2019; 34(1):117-123.

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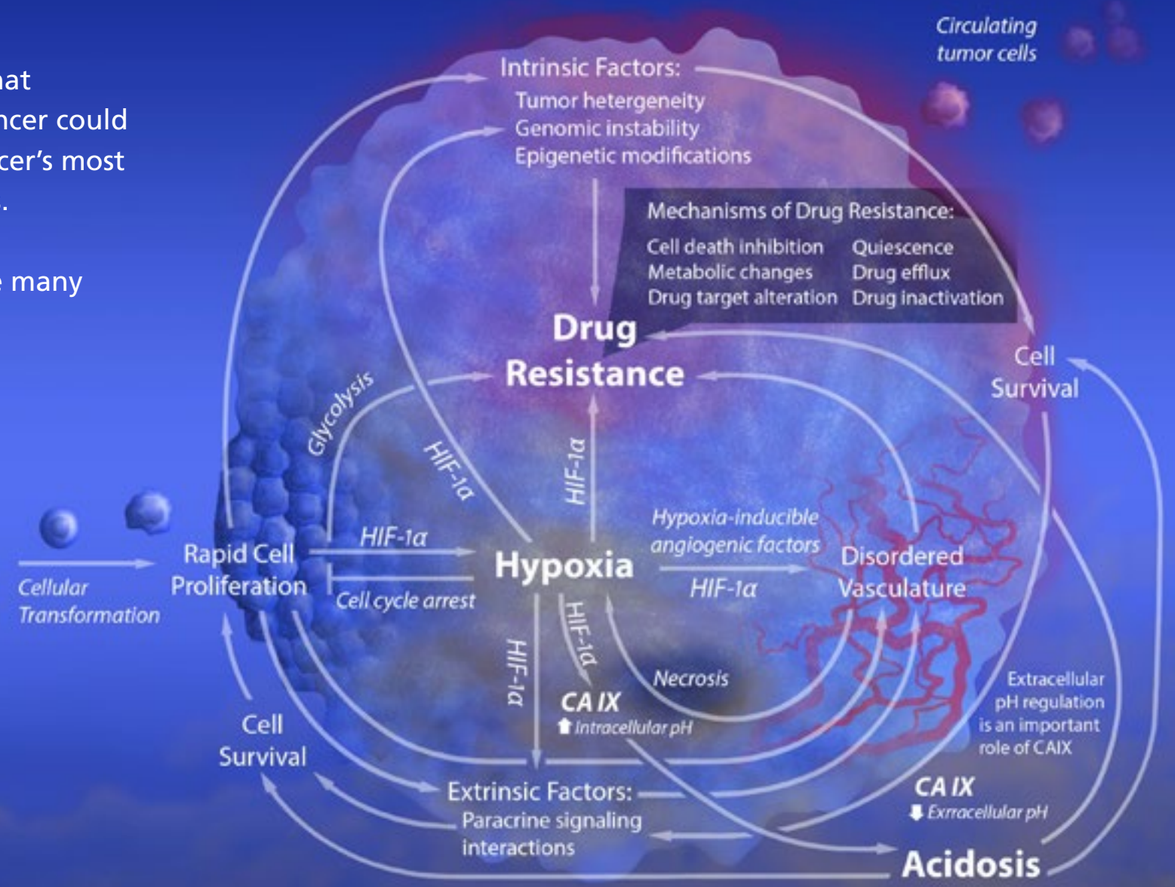
ORIGINS OF THERAPEUTIC RESISTANCE

Unraveling the mechanisms that underlie drug resistance in cancer could potentially disable one of cancer's most significant biological defenses.

This graphic demonstrates the many routes that can lead to drug resistance, and the feedback loops that can support it.



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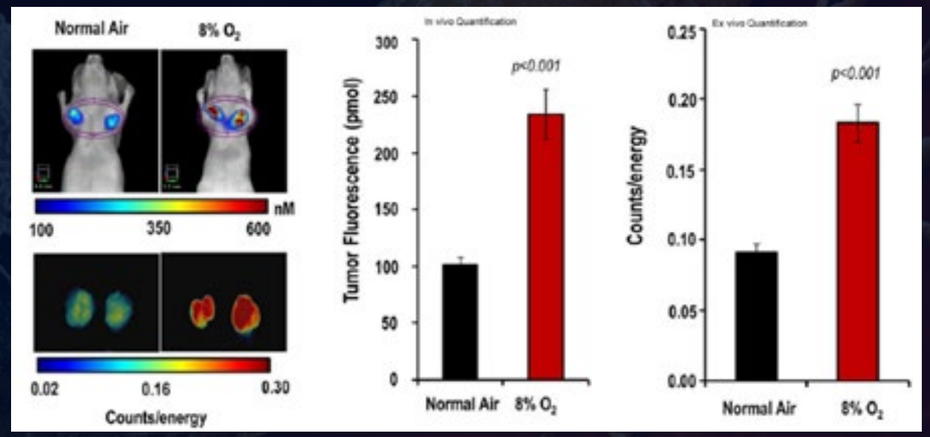
IMAGING CA IX AS A MARKER FOR HYPOXIA IN SMALL TUMORS[1]

While most tumors are expected to have some regions of hypoxia, quantifying increased expression of carbonic anhydrase IX (CA IX) in relatively small tumors, which are reported to be less hypoxic, may present a challenge.

However, CA IX expression can be measured in mice in response to forced hypoxia by maintaining the mice in a low oxygen (8% O_2) environment. When subsequently imaged with the CA IX-specific probe IVISense Hypoxia CA IX 680 (HypoxiSense 680), this method was shown to yield readouts that correlated with *ex vivo* images of excised tumor, confirming that changes in CA IX expression levels as a result of lower oxygen levels, even in small tumors, can be quantified non-invasively by fluorescence imaging.

By imaging *ex vivo* tissue sections, IVISense Hypoxia CA IX 680 was shown to be specifically localized in hypoxic regions of the tumors. These results also indicate the efficacy of Hypoxia CA IX 680 to pene-trate poorly vascularized tumor tissue, a critical property for a non-invasive hypoxia agent.



The effect of breathing 8% O2 on IVISense Hypoxia CA IX 680 (HypoxiSense 680) tumor signal in HeLa xenografts (Bao, et al).

Left: Two groups of mice (n = 4 per group) were used to assess the effect of oxygen breathing levels on Hypoxia CA IX 680 labeling of small tumors. Top Left shows representative fluorescence molecular tomography 3D images of mice breathing normal air (left) and breathing low oxygen (center) showing the intensity differences of Hypoxia CA IX 680 signals within the tumors.

Right: Quantitative analysis of Hypoxia CA IX 680 tumor signals in mice breathing normal or low oxygen, showing greater tumor Hypoxia CA IX 680 signals in mice breathing low oxygen than that of mice breathing normal air.

1. Bao B, Groves K, Zhang J, Handy E, Kennedy P, Cuneo G, et al. (2012) *In Vivo* Imaging and Quantification of Carbonic Anhydrase IX Expression as an Endogenous Biomarker of Tumor Hypoxia. *PLoS ONE* 7(11): e50860.

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CARBONIC ANHYDRASE IX

Carbonic anhydrase IX (CA IX) is a transmembrane cell surface enzyme that catalyzes the reversible conversion of CO_2 into bicarbonate and a proton. Normally expressed in only a few human tissues, CA IX is a tumor-associated protein that serves as an important biomarker in the study of both hypoxia and acidosis in tumor progression and response to drug therapy.

Expression of CA IX is primarily regulated at the transcriptional level by hypoxia-inducible factor-1α (HIF-1α).[1] When expressed, CA IX helps produce and maintain an intracellular pH favorable for cell growth and survival, while at the same time contributing to an increasingly acidic extracellular space (acidosis). This not only facilitates tumor progression and invasiveness, but also promotes conditions for cells to develop mechanisms of drug resistance such as epigenomic and genomic changes, metabolic changes, drug inactivation and drug efflux capabilities, quiescence, and cell death inhibition.

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1. Tafreshi NK, Lloyd MC, Bui MM, Gillies RJ, Morse DL. Carbonic anhydrase IX as an imaging and therapeutic target for tumors and metastases. *Subcell Biochem*. 2014;75:221-254.

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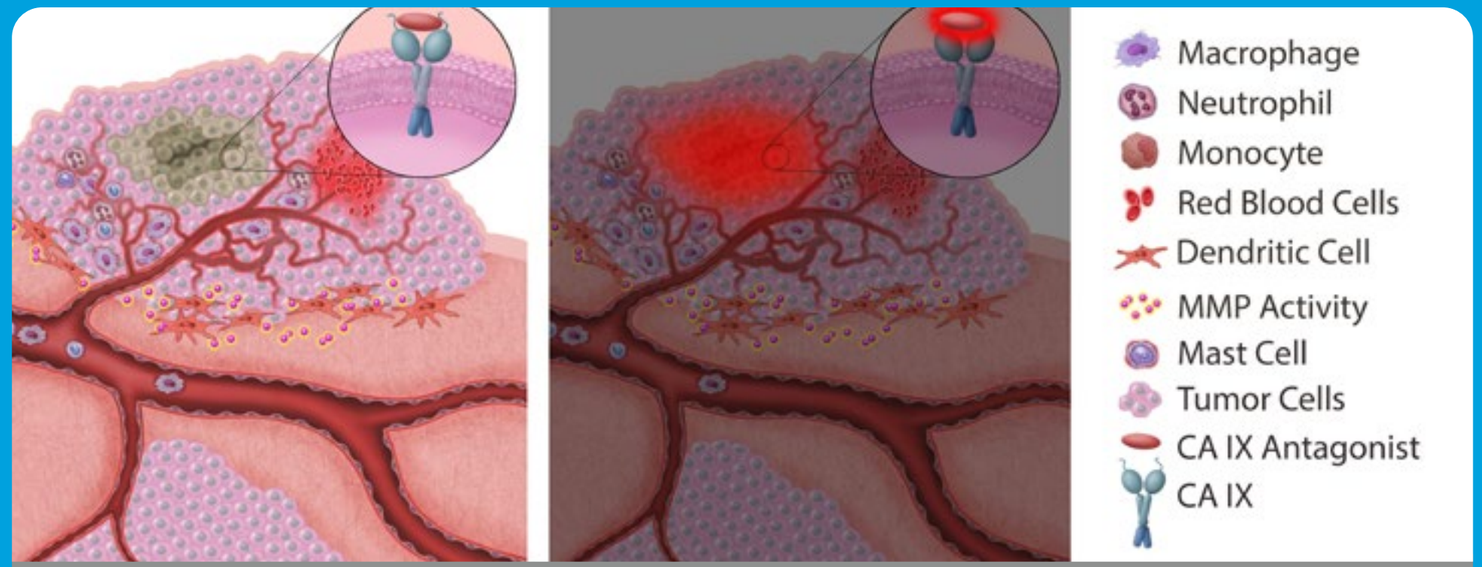
CARBONIC ANHYDRASE IX



Carbonic Anhydrase IX (CA IX) is highly expressed in tumor cells in response to a low oxygen environment, yielding significant intracellular pH changes and extracellular acidosis to favor cancer survival. IVISense Hypoxia CA IX fluorescent imaging of hypoxic regions is represented on the right.

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CA IX EXPRESSION[1]

Present in few normal tissues, CA IX expression is mainly limited to the stomach and gallbladder epithelia. It is upregulated in some but not all cancer cell lines, while very often being highly expressed in spontaneously arising, generally more aggressive tumors.

Among those known to upregulate CA IX expression are CNS, head & neck, lung, breast, colon, cervical, ovarian, prostate, renal cancers, leukemia, and melanoma. In addition, a variety of other tumors show increased focal expression of CA IX, occurring in 90% of cervical, glioblastoma, and basal cell carcinomas, as well as in 25– 30% of non-invasive and invasive ductal breast cancers.

1. Bao B, Groves K, Zhang J, Handy E, Kennedy P, Cuneo G, et al. (2012) *In Vivo* Imaging and Quantification of Carbonic Anhydrase IX Expression as an Endogenous Biomarker of Tumor Hypoxia. *PLoS ONE* 7(11): e50860.

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CASE STUDY: IMAGING DOWNSTREAM BIOLOGY TO INTERROGATE A NEW THERAPEUTIC TARGET FOR TREATING GLIOMA[1]

In glioma, hypoxia is considered the major driving force for tumor angiogenesis. Since inhibiting angiogenesis impairs tumor progression, understanding the molecular players involved in hypoxia-mediated angiogenesis could yield novel therapies.

The cellular response to low oxygen concentrations is regulated by hypoxia-inducible factors (HIFs). In hypoxic cancer cells, the HIF pathway is up-regulated, including HIF-1α the master gene for cellular response to hypoxia. Under normoxic conditions, HIF-1αis rapidly degraded. However, under hypoxic conditions, it can escape proteolysis, altering a multitude of downstream genes to promote cellular survival and proliferation. Among those genes is the long non-coding RNA (lncRNA) H19, in which the microRNA, miR675-5p, is embedded. Another gene expressed downstream from HIF-1αis carbonic anhydrase IX (CA IX) which has been shown to be a biomarker of HIF-1αactivity.

The authors here demonstrate that miR675-5p is a hypoxia-regulated microRNA able, by itself, to mimic a low oxygen condition in normoxia, and was found to be: i) essential for hypoxia establishment, ii) a stimulator of hypoxia-mediated angiogenesis and iii) a promoter of glioma progression.

As part of this demonstration, the authors generated a glioma orthotropic murine model using U251-HRE-mCherry cells. The mice were divided into two groups treated with either an miRNA675-5p mimic or a scramble. The mice were imaged to detect mCherry fluorescence to indicate hypoxia, and fluorescent probes to quantify two biomarkers; CA IX which indicates HIF-1αexpression, and the neo-angiogenesis biomarker αvβ3 integrin which indicates neoangiogenesis. Using IVISense Hypoxia CA IX (HypoxiSense) and IVISense Integrin Receptor (IntegriSense) probes respectively, image acquisitions showed the induction of CA IX expression in mimic mice as well as increased αvβ3 integrin expression. These findings were in agreement with increased hypoxia as indicated by mCherry fluorescence, and an increase in HIF-1 activity and neo-angiogenesis stimulation, as shown by immunohistochemistry and CT scan.

Mice were subsequently treated with an inhibitor of miRNA675-5p and imaged in the same way. The data indicate that the inhibition of miRNA675-5p *in vivo* can i) turn off tumor molecular response to hypoxia ii) constrain angiogenesis and iii) block hypoxia-mediated tumor growth, thus suggesting a therapeutic role for miRNA675-5p inhibitor in glioblastoma.
See figures

1. Lo Dico A, Costa V, Martelli C, Diceglie C, Rajata F, Rizzo A, Mancone C, Tripodi M, Ottobrini L, Alessandro R, Conigliaro A. MiR675-5p Acts on HIF-1αto Sustain Hypoxic Responses: A New Therapeutic Strategy for Glioma. *Theranostics*. 2016 May 8;6(8):1105-18.

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- Hydroxyapatite

CELL DEATH
- Transferrin

RESOURCES

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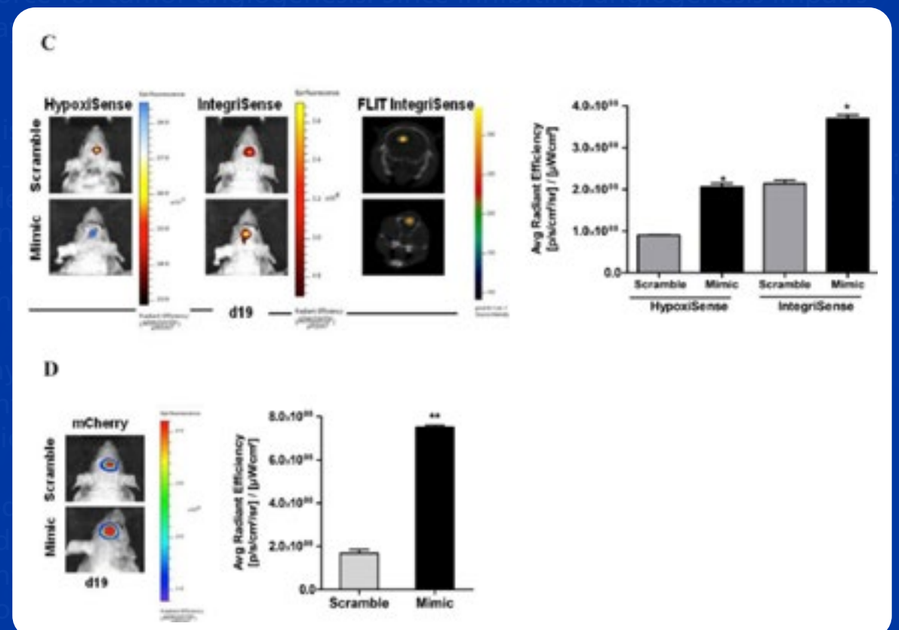
FIGURE 1 | FIGURE 2

miR675-5p *in vivo* is able to enhance hypoxia establishment and neo-angiogenesis.



(C) Left panel: Representative 2D images of mice co-injected with IVISense Hypoxia CA IX 680 (HypoxiSense, BlueHot scale) and IVISense Integrin Receptor 750 (IntegriSense, YellowHot scale) and axial images of IVISense Integrin Receptor 750 co-registered with CT scan. All scans were performed at the final time point (day 19).

(C) Right panel: Quantification of IVISense Hypoxia CA IX 680 and IVISense Integrin Receptor 750 fluorescence by ROI analysis for scramble and mimic-treated mice. Data are expressed as average radiance efficiency [(photons/s/cm^2/steradian)/(µW/cm^2)].

(D) Left panel: Representative (n=5) 2D mCherry fluorescent signal (rainbow scale) of U251-HRE-mCherry tumors in scramble and miR675-5p mimic-treated mice at day 19.

(D) Right panel: Graphical representation of mCherry activity at the end of treatment. Data are presented as average radiance efficiency [(photons/s/cm^2/steradian)/(µW/cm^2). Images are presented with the same scale bar. Mimic-treated mice vs scramble- treated mice * p<0.05; **p<0.01; ***p<0.001.

1. Lo Dico A, Costa V, Martelli C, Diceglie C, Rajata F, Rizzo A, Mancone C, Tripodi M, Ottobrini L, Alessandro R, Conigliaro A. MiR675-5p Acts on HIF-1αto Sustain Hypoxic Responses: A New Therapeutic Strategy for Glioma. Theranostics. 2016 May 8;6(8):1105-18.

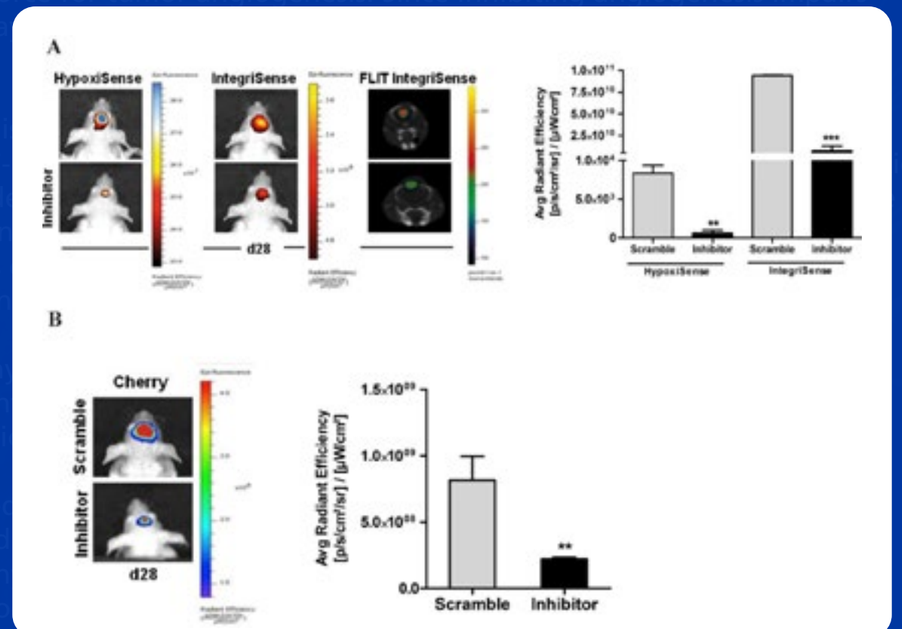
FIGURE 1

FIGURE 2

miR675-5p inhibitor counteracts hypoxia-mediated angiogenesis *in vivo*.

(A) Left Panel: Representative (n=5) 2D images of scramble or miR675-5p inhibitor-treated mice co-injected with IVISense Hypoxia CA IX 680 (HypoxiSense, BlueHot scale) and IVISense Integrin Receptor 750 (IntegriSense, YellowHot scale) and axial images of IVISense Integrin Receptor 750 co registered with CT scan. All scans were performed at the final time point (day 28).

(A) Right panel: Quantification of IVISense Hypoxia CA IX 680 and IVISense Integrin Receptor 750 fluorescence by ROI analysis for scramble and miR675-5p inhibitor-treated mice (considering five mice for each treatment). Data are expressed as average radiance efficiency [(photons/s/cm^2/steradian)/(μW/cm^2)].



(B) Left Panel: Representative (n=5) 2D mCherry fluorescent signal (rainbow scale) of U251-HRE-mCherry tumors in scramble and inhibitor-treated mice (one of five mouse for each treatment) at day 28. Images are presented with the same scale bar.

(B) Right panel: Graphical representation of mCherry activity at the end of treatment considering five mice for each treatment. Data are presented as the average radiance efficiency [(photons/s/cm^2/steradian)/(μW/cm^2). Images are presented with the same scale bar. Inhibitor-treated mice vs scramble-treated mice * p<0.05; **p<0.01; ***p<0.001.

TUMOR BURDEN
METABOLIC ACTIVITY
ANGIOGENESIS
METASTASIS
DRUG RESISTANCE
INFLAMMATION
CELL DEATH

Folate
MMP
Cathepsin
Carbonic Anhydrase IX
Phosphatidylserine
Endothelium
αvβ3 Integrin
Bombesin
Elastase
Hydroxyapatite
Transferrin

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BY CHARACTERIZATION

IVISense Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25 g)	Sold As (Unit Size)	Optimal Imaging Time (h)
Hypoxia CA IX 680	1,500	Carbonic Anhydrase IX	Detection of carbonic anhydrase 9 (CA IX) protein expressed in many tumor cell lines undergoing hypoxia and acidosis.	2 nmol	24 nmol	24

Targeted probes actively target and bind to distinct biomarkers with highly specific targeting to key biological mechanisms.

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TUMOR BURDEN
- Folate

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The current view of inflammation during carcinogenesis is a complex dichotomy of opposing processes: immunosurveillance and anti-tumorigenic immune functioning are countered by pro-tumorigenic inflammation, which promotes cancer by blocking anti-tumor immunity and shaping the tumor microenvironment toward a more tumor-permissive state.[1] Additionally, inflammation can both contribute to and be driven by tumor growth. It is well known that long-standing inflammation that is secondary to chronic infection or repeated tissue insult can predispose to cancer.[2] The reverse is also true, where "wound-like" pre-malignant tumors trigger a tissue repair response, eliciting pro-inflammatory factors which in later stages of tumor growth can function to promote cancer under direct control of the tumor itself.[2]

Regardless of its initiation, long-term 'smoldering' inflammation in the tumor microenvironment has many tumor-promoting effects, such as aiding in the proliferation and survival of malignant cells, promoting angiogenesis and metastasis, subverting adaptive immune responses, and altering responses to hormones and chemotherapeutic agents.[3]

A deeper understanding of the role of cancer-related inflammation, including the identification of new targets and more effective approaches to mediating the inflammatory response, is central to the development of improved diagnosis and novel treatments.

1. Greten FR, Grivennikov SI. Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity*. 2019; 51(1):27-41.
2. Rakoff-Nahoum S. Why cancer and inflammation? *Yale J Biol Med*. 2006; Dec;79(3-4):123-30.
3. Mantovani, A., Allavena, P., Sica, A. et al. Cancer-related inflammation. *Nature*. 2008; 454: 436–444.

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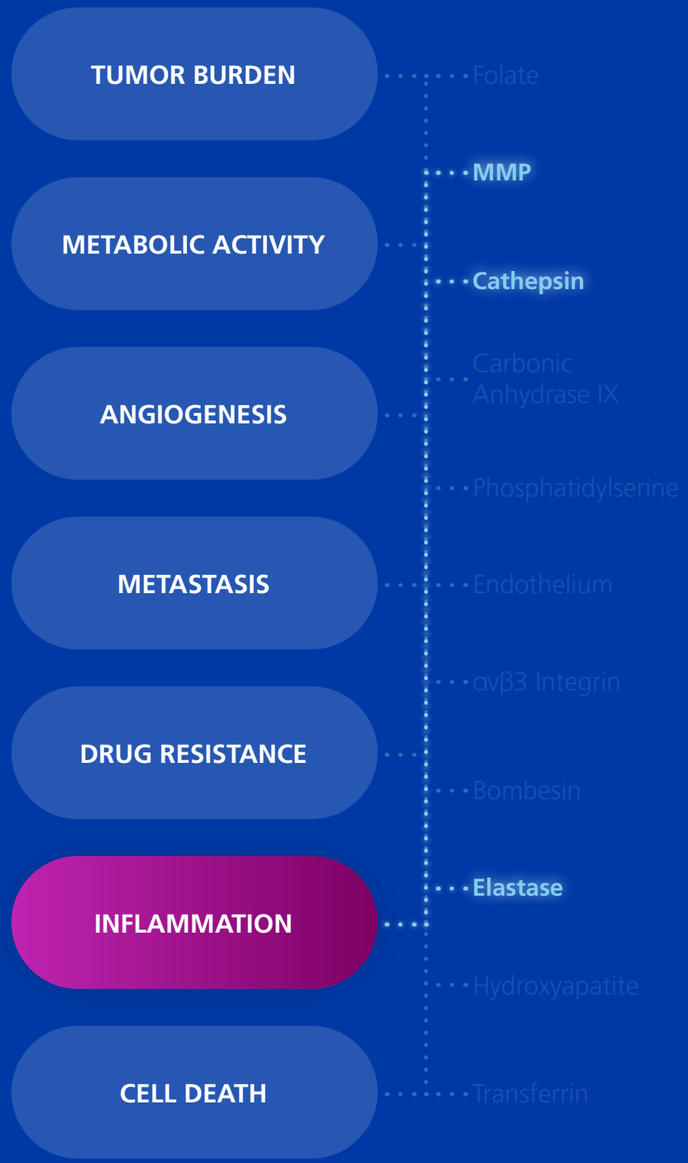

A critical biological consideration with *in vivo* imaging is the timing of a particular biomarker's activity. Disease biomarkers may be up- or down-regulated at different times, as different factors may influence their expression during disease progression. Thus, understanding when the biomarker is operant so that the corresponding imaging probe can be optimized is paramount. A useful example of probes scheduled to longitudinally detect mutable inflammatory markers was devised based on each probes' potential use in imaging atherosclerosis in ApoE-deficient mice. It is worth noting that atherosclerotic plaques start in much the same way as many cancers, and the link in patterns of progressive inflammation is currently being investigated.

Atherosclerosis and cancer are both chronic, multifactorial diseases, sharing not only several important molecular pathways but also many etiological and mechanistical processes from the very early stages of development up to the advanced forms in both pathologies.[1] In atherogenesis, small sub-endothelial deposits of monocyte-derived macrophages cause tissue insult which then drives an inflammatory cascade, recruiting additional macrophages and further propagating the inflammatory process. This initiating tissue insult, along with other factors such as uncontrolled cell proliferation and oxidative stress, bares considerable similarities to the neoplastic process of cancer.[1]

Research with the aim to identify molecular ties between the phenomenon that closely correlates atherosclerosis and cancer is ongoing, with the hope that this new understanding could lead to novel treatments, such as a cancer treatment currently in clinical trials which may also reduce the inflammation responsible for atherosclerosis.[2]

RESOURCES

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1. Tapia-Vieyra JV, Delgado-Coello B, Mas-Oliva J. Atherosclerosis and Cancer; A Resemblance with Far-reaching Implications. *Archives of Medical Research*, 2017; 48(1): 12-26.
2. Jarr K, et al. Effect of CD47 Blockade on Vascular Inflammation. *N Engl J Med*, 2021; 384:382-383.

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PROBE USE IN A LONGITUDINAL IMAGING STUDY: POTENTIAL IMAGING OF DISEASE PROGRESSION IN APOE-DEFICIENT MICE FED HIGH CHOLESTEROL DIET (HCD)[1]

Time on HCD (weeks)	Concurrent Biology[2]	IVISense Probe						
		Pan Cathepsin (ProSense)	MMP (MMPSense)	Cat B FAST	MMP FAST (MMPSense FAST)	Integrin Receptor (IntegriSense)	Annexin-V (Annexin-Vivo)	Cat K FAST
4-6	• Moncyte adhesion • Sporadic foam cells							
8-10	• Early foam cell lesions • Subendothelial progression			■				
10-15	• Intermediate lesions • Smooth muscle cell proliferation			■	■	■		
15	• Larger fibrous plaques • Small necrotic cores		■	■	■	■	■	
20-30	• Advanced plaques • Increased necrosis	■	■	■	■	■	■	
30-40	• Larger, advanced plaques • Progression to coronary arteries • Larger necrotic cores • Partial medial destruction	■	■	■	■	■	■	■

Relevant Targets:

- ■ Broad or selective (Cat B, Cat K) cathepsin activity
- ■ Matrix metalloprotease (MMP) activity
- ■ αVβ3 integrin expression
- ■ Apoptosis (phosphatidyl-serine)

1. Peterson, JD. Non-Invasive Quantitative In Vivo Imaging of Atherosclerosis Disease Progression and Treatment Response in ApoE Deficient Mice using Fluorescent Molecular Tomography and NIR Fluorescent Pre-clinical Imaging Agents. *Application Note*. PerkinElmer, Inc. Waltham, MA.
2. Nakashima et al., ApoE-deficient Mice Develop Lesions of All phases of Atherosclerosis Throughout the Arterial Tree, *Arterioscler Thromb* 1994, 14(1):133-40.

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MMP | CATHEPSIN | ELASTASE

Matrix metalloproteases (MMPs) are known to cleave numerous cell surface proteins in remodeling the extracellular matrix but have been shown to also have a role in modulating the immune response via their proteolytic regulatory functions. Expressed at low levels under homeostasis, MMP expression is markedly increased in inflammatory and cancer cells during inflammatory processes including infection, wound healing, and cancer. Increasing research is showing that MMPs are not just a downstream consequence of a generalized inflammatory process, but rather are critical factors in the overall regulation of the pattern, type, and duration of immune responses.[1]

The ability to detect MMPs can reveal distinctive local biology as well as assess therapeutic efficacy of specific drugs, sometimes prior to overt biological changes. IVISense MMP NIR fluorescent imaging probes are able to detect a broad range of active MMPs, including MMP 2, 3, 7, 9, 12, and 13, to evaluate the potential therapeutic efficacy of drugs targeting the underlying mechanisms involved.

VIEW IMAGE ▶

1. Smigiel KS, Parks WC. Matrix Metalloproteinases and Leukocyte Activation. *Prog Mol Biol Transl Sci*. 2017;147:167-195.

TUMOR BURDEN
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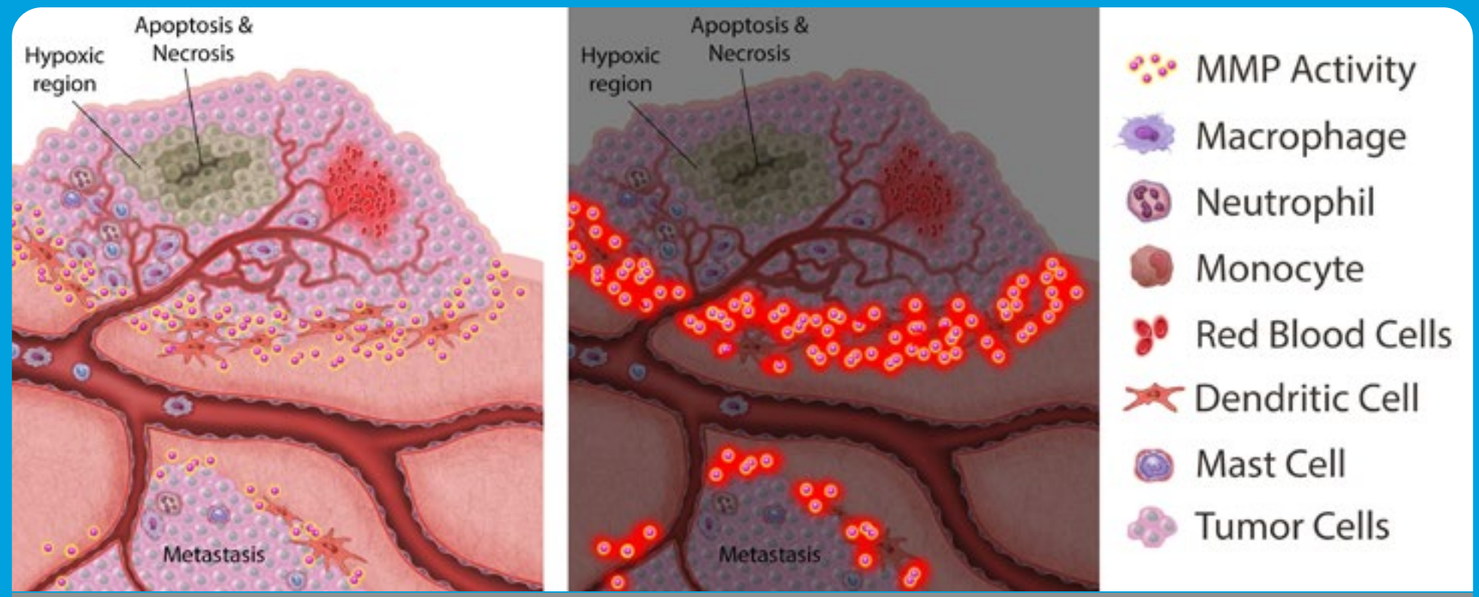
MMP | CATHEPSIN | ELASTASE



Matrix metalloproteases are active in regions of tissue remodeling, in this case highly expressed and secreted at the invasive tumor margins as well as by inflammatory cells.

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MMP | **CATHEPSIN** | ELASTASE

Cathepsins are a family of proteases that are pivotal components of signaling pathways which can enhance cancer cell growth and inflammation, and contribute to matrix degradation, thereby facilitating tumor cell invasion.

Cathepsin B: Upregulated in a variety of cancers as well as infiltrating inflammatory cells (including eosinophils, neutrophils, and macrophages), cathepsins are capable of exerting either anti- or pro-cancer effects. They may initiate proteolytic pathways crucial for invasion by inflammatory breast cancer, the most lethal form of primary breast cancer.[1]

Cathepsin K: A lysosomal cysteine protease involved in bone resorption and collagen degradation, and expressed in osteoclasts, certain tumor-associated macrophages (TAMs), and other cells, such as bone marrow macrophages which are known to mediate the inflammatory process in the bone microenvironment.

IVISense Pan Cathepsin fluorescent imaging probes: versatile pan-cathepsin-activated agents.
IVISense Cat B fluorescent imaging probes: selectively cleaved by cathepsin B proteinases.
IVISense Cat K fluorescent imaging probes: selectively cleaved by cathepsin K proteinases.

For specific detection of tumors with associated cathepsin activity, NIR fluorescent imaging agents ProSense, Cat B, and Cat K enable non-invasive longitudinal imaging of tumor progression via protease expression.

VIEW IMAGE ▶

1. Nouh, M.A., Mohamed, M.M., El-Shinawi, M. et al. Cathepsin B: a potential prognostic marker for inflammatory breast cancer. *J Transl Med*. 2011; 9(1)

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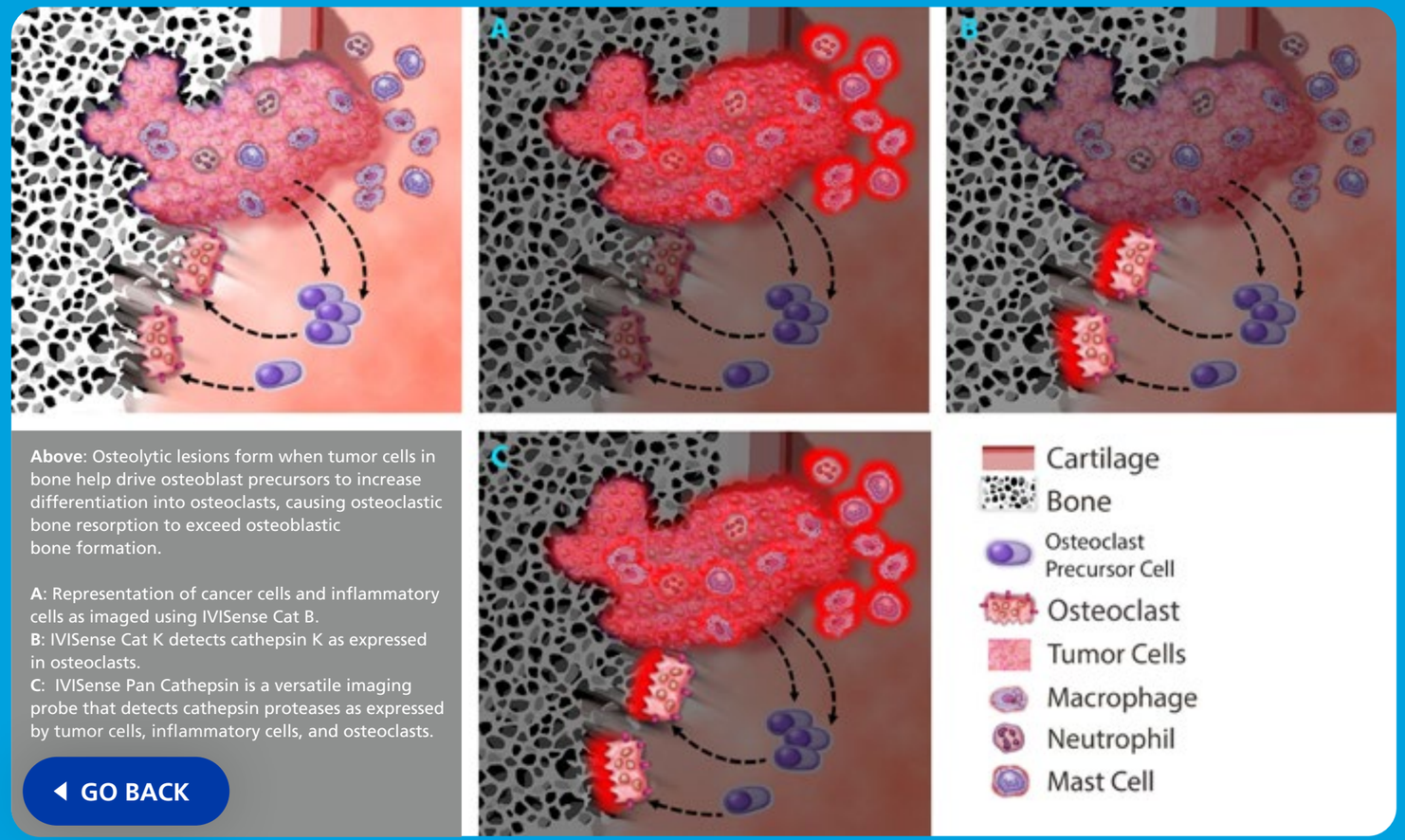

Above: Osteolytic lesions form when tumor cells in bone help drive osteoblast precursors to increase differentiation into osteoclasts, causing osteoclastic bone resorption to exceed osteoblastic bone formation.

A: Representation of cancer cells and inflammatory cells as imaged using IVISense Cat B.
B: IVISense Cat K detects cathepsin K as expressed in osteoclasts.
C: IVISense Pan Cathepsin is a versatile imaging probe that detects cathepsin proteases as expressed by tumor cells, inflammatory cells, and osteoclasts.

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MMP | CATHEPSIN | ELASTASE

Neutrophil elastase (NE), a major product of neutrophils, is an especially potent proteolytic enzyme, normally stored in vesicles in an inactive form prior to degranulation or to the release of neutrophil extracellular traps (NETs), a process of cell death called NETosis whereby a network of extracellular fibers dotted with NE as arsenal against an injurious agent is released from the cell. Extensively studied as bacterial-responsive, expendable innate immune cells, neutrophils have recently been found to be more heterogeneous than was traditionally thought and to possess a longer survival time[1], sparking renewed interest in the study of neutrophil biology.

Neutrophils are frequently found within tumors, prompting adoption of the term 'tumor-associated neutrophil' (TAN). TANs are less well characterized than their counterpart, 'tumor-associated macrophages' (TAMs), but have been shown to have both pro- and anti-tumor effects.[2] Found to perform previously unsuspected tasks, neutrophil functionality may include modulating the activity of other immune cells, such as either boosting or suppressing T-cell activation. Multiple other tumor-promoting functions of TANs have been described, such as encouraging cellular proliferation, tumor angiogenesis, metastasis, and immunosuppression.[2]

IVISense Neutrophil Elastase 680 FAST is a near infrared fluorescent *in vivo* imaging probe that can detect NE post-degranulation or NETosis. (See Biomarker Considerations for further discussion of the *in vivo* study of tumor-associated neutrophil activity.)

VIEW IMAGE ▶

1. Carlos R. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? *Frontiers in Physiology*. 2018; Vol. 9
2. Elliot A, Myllymaki H, Feng Y. Inflammatory Responses during Tumor Initiation: From Zebrafish Transgenic Models of Cancer to Evidence from Mouse and Man. *Cells*. 2020;9(4) 1018

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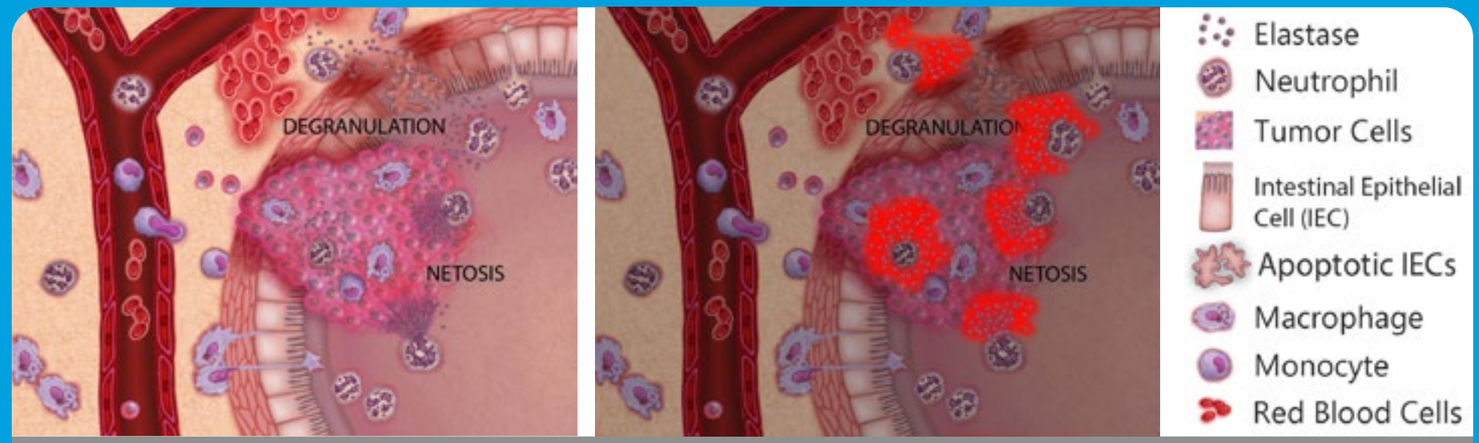

Tissue damage from inflammatory bowel disease can lead to abnormal cell growth with the potential to become invasive cancer. Neutrophil Elastase activation by extracellular NE and NETs is represented in the figure on the right.

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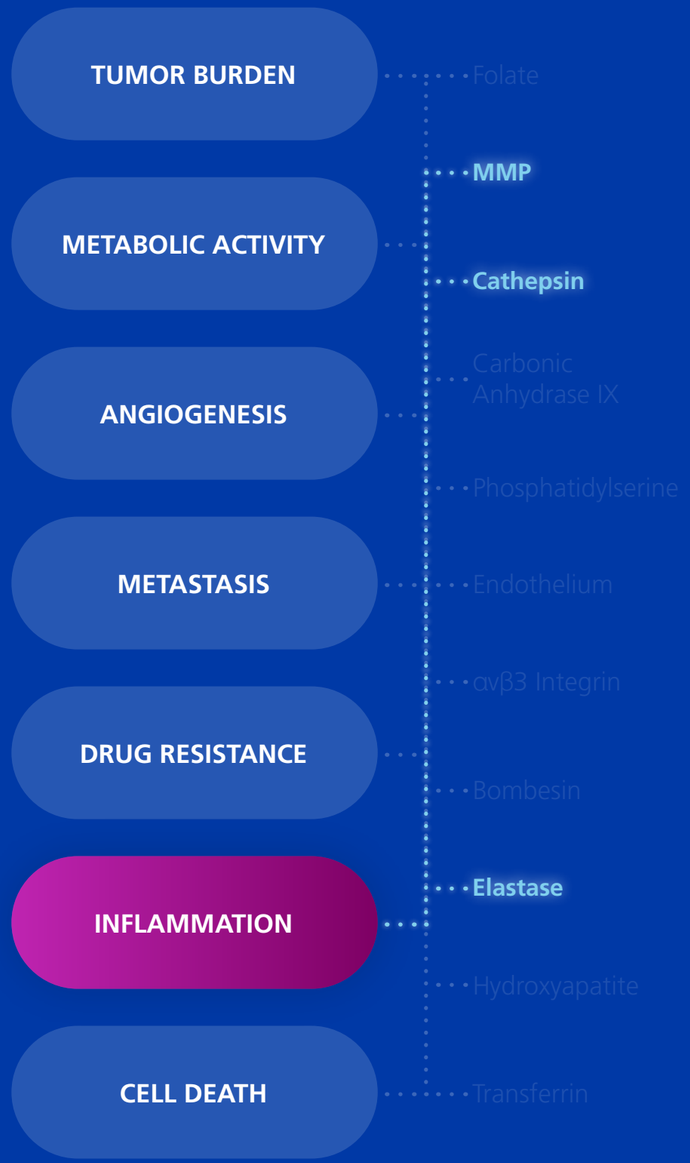

Neutrophils play an important role in shaping the tumor microenvironment. Upon degranulation, they release enzymes including neutrophil elastase (NE) which significantly promotes cancer cell proliferation, metastasis, and therapy resistance.[1]

A well-designed *in vivo* study of neutrophils requires a full understanding of the animal model and the kinetics of degranulation and NETosis as biomarkers of neutrophil activity. It is important to note that there are situations in which neutrophils are present, but degranulation has not yet occurred. In some models, neutrophil degranulation is a coordinated occurrence, while in others the degranulation is sporadic. This means one should plan their study design carefully, as it can be easy to miss the optimal imaging time for NE activity.

Apart from neutrophils, cells such as tumor cells, B cells, lymphocytes, and macrophages present in the tumor microenvironment also secrete regulatory factors to facilitate cancer progression. Monitoring neutrophils as part of an imaging study to monitor the overall innate immune response may provide a true sense of the ratio of activated neutrophils to other immune cells involved. This could help researchers gain a deeper understanding of the pathological process of the inflammatory response, with the ultimate goal of regulating neutrophil recruitment for better clinical outcomes.

IVISense Neutrophil Elastase 680 FAST is a near infrared fluorescent *in vivo* imaging probe that can detect NE post-degranulation or NETosis. A highly specific probe, it can detect active NE in the extra cellular matrix.

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1. Wu L, Saxena S, Awaji M, Singh RK. Tumor-Associated Neutrophils in Cancer: Going Pro. *Cancers (Basel)*. 2019;11(4):564.

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CASE STUDY: IMAGING THE EFFECTS OF N-ACETYLCYSTEINE (NAC) ANTIOXIDANT ON CHRONIC INFLAMMATORY PATHOLOGY RESULTING FROM EPSTEIN-BARR VIRUS[1]

Numerous studies have established a link between chronic inflammation and increased cancer risk. When the inflammatory response is persistently activated or fails to resolve, chronic inflammation can result, leading to tissue damage and promoting conditions favorable for cancer development. Certain viral infections can induce persistent inflammation, generating high levels of reactive oxygen and nitrogen species which then perpetuate the inflammatory response and cause damage to a wide range of biomolecules. One such virus is the Epstein-Barr virus (EBV) which commonly results in chronic inflammatory post-viral disease.

In this study, the effects of long-term administration of NAC, an antioxidant involved in redox regulation, on chronic inflammation was investigated. Specifically, researchers used a transgenic mouse model of chronic inflammation-associated carcinogenesis induced by the viral-encoded latent membrane protein 1 (LMP1) of EBV. LMP1 acts as a pro-inflammatory factor with multiple oncogenic properties, including the ability to activate proliferative, cell survival, and inflammatory pathways. It was found that treatment with NAC resulted in a significant reduction of leukocyte infiltrate in inflamed tissue, an amelioration of disease effects, and a delay in the chronic progressive inflammatory phenotype as quantified by *in vivo* imaging using IVISense Pan Cathepsin 680 (ProSense 680).

Given that targeting any one chemokine or cytokine in the inflammatory process may have limited effect and/or unwanted side effects, the researchers instead sought to assess the ability of a readily available, non-toxic antioxidant to inhibit the pernicious cycle of inflammation, oxidative stress, and tissue damage, showing that NAC could be considered as a treatment to alleviate chronic inflammatory pathologies, including post-viral disease. While the use of antioxidants in cancer treatment and prevention is much debated and the effects are likely to be cancer-type dependent, this study supports the idea that NAC in the treatment of inflammation-associated and specifically EBV/LMP1-associated cancers could be a valid area for further exploration.

See figures

1. Gao X, Lampraki EM, Al-Khalidi S, Qureshi MA, Desai R, et al. N-acetylcysteine (NAC) ameliorates Epstein-Barr virus latent membrane protein 1 induced chronic inflammation. 2017; *PLoS ONE* 12(12).

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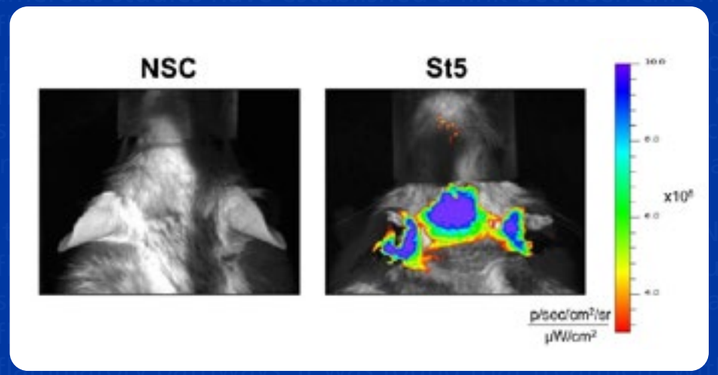
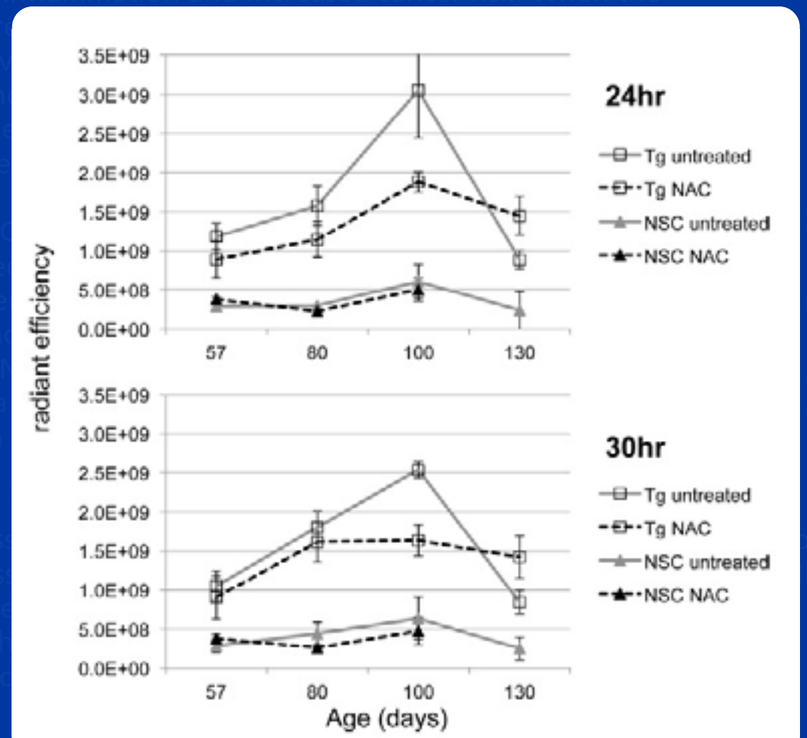


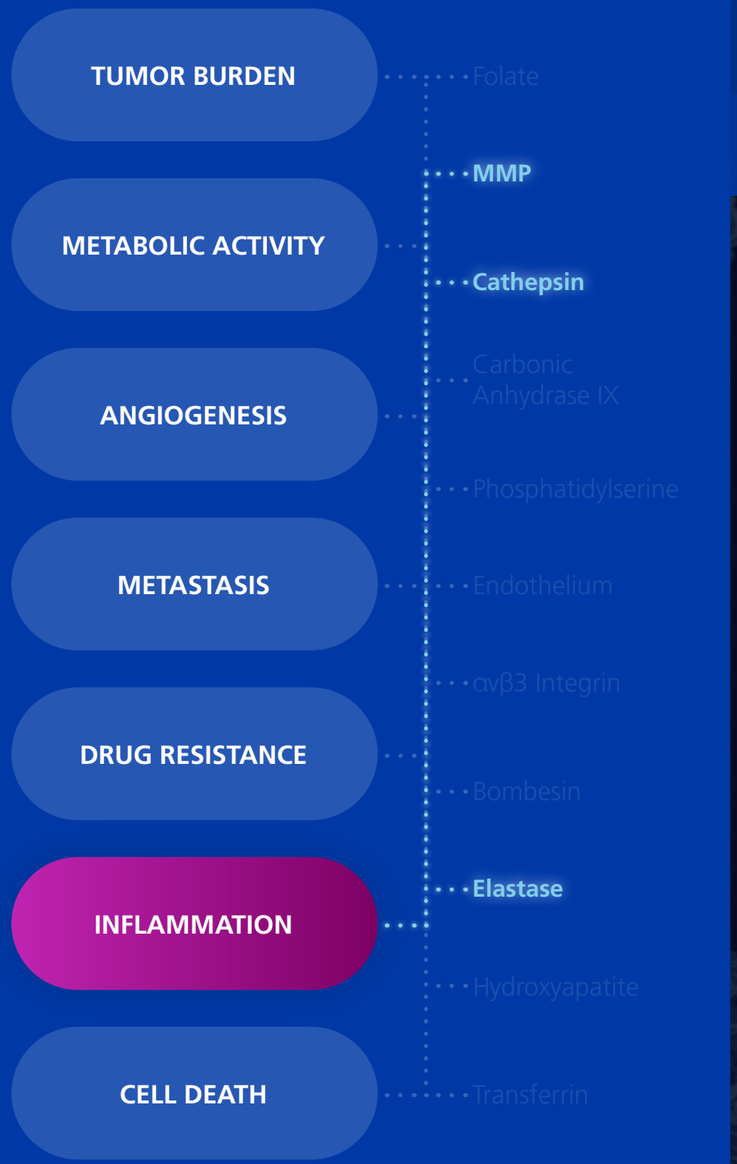


IVISense Pan Cathepsin 680 was used to show N-acetylcysteine (NAC) treatment reduces the quantifiable levels of chronic inflammation in the L2LMP1 transgenic mice.

Left: Typical epifluorescent image taken 24 hours after IV injection of IVISense Pan Cathepsin 680 into a transgenic L2LMP1 mouse at phenotypic stage 5 (St5) and negative sibling control (NSC); color scale shows radiant efficiency range displayed ((photons/sec/cm2/sr)/(µW/cm2)).

Right: Graphs depicting the mean (with SD error bars) radiant efficiency (Y axis) observed in mice in increasing age groups (X axis) imaged at 24 and 30 hours (as indicated) post injection of 1nmol/mouse IVISense Pan Cathepsin 680. Four groups were examined, transgenic (Tg) and NSC mice, either untreated or treated with NAC. N = 2 to N = 8 for each age and group.

1. Gao X, Lampraki EM, Al-Khalidi S, Qureshi MA, Desai R, et al. N-acetylcysteine (NAC) ameliorates Epstein-Barr virus latent membrane protein 1 induced chronic inflammation. 2017. *PLOS ONE* 12(12).



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BY CHARACTERIZATION | BIOPROFILING

IVISense Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25 g)	Sold As (Unit Size)	Optimal Imaging Time (h)
✂ Cat B 680 FAST	33,000	Cathepsin B	Selectively cleaved by cathepsin B proteinases upregulated in tumors and inflammatory cells	2 nmol	24 nmol	6-24 h
✂ Cat B 750 FAST	23,000			4 nmol	48 nmol	6-24 h
✂ Cat K 680 FAST	8,500	Cathepsin K	Selectively cleaved by cathepsin K proteinases upregulated in osteoclasts and tumor associated macrophages.	2 nmol	24 nmol	6-24 h
✂ Pan Cathepsin 680	~400,000	Cathepsin proteases	Pan-cathepsin activatable agent that detects abnormal upregulation of cathepsin secretion associated with cancer and inflammation.	2 nmol	20 nmol	24 h (24-48)
✂ Pan Cathepsin 750	~450,000			2 nmol	24 nmol	24 h
✂ Pan Cathepsin 750 FAST	22,500			4 nmol	48 nmol	6-24 h
✂ MMP 645 FAST	43,000	Matrix Metalloprotease	Cleaved by multiple metalloproteinases associated with many disease areas including cancer and inflammation.	4 nmol	48 nmol	24 h (6-24)
✂ MMP 680	~450,000			2 nmol	20 nmol	24 h (24-36)
✂ MMP 750 FAST	43,000			2 nmol	24 nmol	24 h (12-24)
✂ Neutrophil Elastase 680 FAST	43,000	Neutrophil Elastase	Selectively cleaved by elastase produced by activated neutrophils during acute inflammation.	4 nmol	48 nmol	3-6 h

✂ Activatable probes are optically silent upon injection and become highly fluorescent following protease-mediated activation. Activatable FAST agents are designed with a novel small molecule architecture that confers an accelerated pharmacokinetic profile with earlier imaging time points.

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BY CHARACTERIZATION | BIOPROFILING

PerkinElmer offers a fluorescent Inflammation Panel consisting of a carefully curated collection of probes to detect multiple aspects of inflammation. Including probes at various wavelengths (680nm and 750nm), the panel offers the opportunity for multiplex imaging of appropriate probe combinations to maximize information gained from your research results.

Inflammation Panel
Set of Fluorescent Probes Bundled by Application

IVISense Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25 g)	Amount (Unit Size)	Optimal Imaging Time (h)	Probe Clearance
Vascular 680	~70,000	NA	Imaging of vascularity, perfusion, and vascular permeability. Imaging of vasculature for up to 4 h; tumor accumulation at 24 h.	2 nmol	24 nmol	24 h	6-7 d
Folate Receptor 680	1,606	Folate Receptor	Detection of Folate Receptor protein upregulation for monitoring tumor growth and metabolism.	2 nmol	24 nmol	6 h (6-24)	6-7 d
Pan Cathepsin 750 FAST	22,500	Cathepsin proteases	Pan-cathepsin activatable agent that detects abnormal upregulation of cathepsin secretion associated with cancer and inflammation.	4 nmol	48 nmol	6-24 h	3 d
MMP 750 FAST	43,000	Matrix Metalloprotease	Cleaved by multiple metalloproteinases associated with many disease areas including cancer and inflammation.	4 nmol	48 nmol	24 h (12-24)	6-7 d
Integrin Receptor 750	1,278	αvβ3 integrin	Detection of integrin αvβ3 expression in tumors and neovasculature.	2 nmol	24 nmol	24 h	4-6 days
Neutrophil Elastase 680 FAST	43,000	Neutrophil Elastase	Selectively cleaved by elastase produced by activated neutrophils during acute inflammation.	4 nmol	48 nmol	3-6 h	2 d

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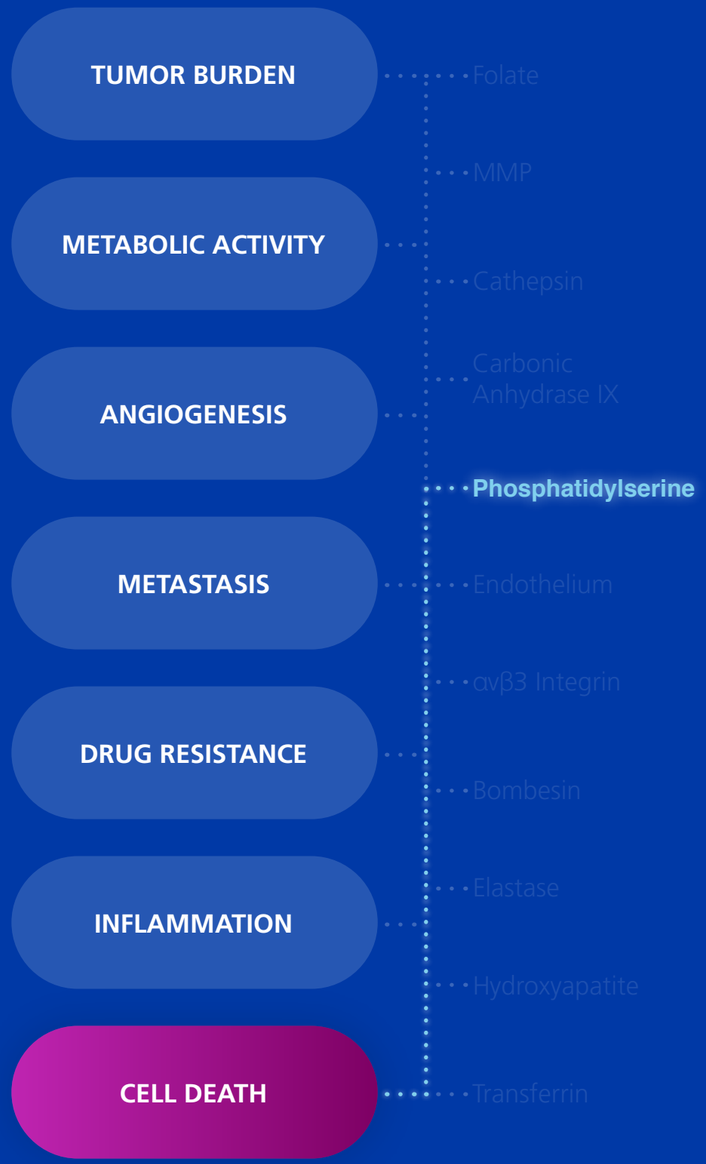

Cell death is a fundamental process in all multicellular organisms. For many years it was considered to occur in one of two basic forms – apoptosis, defined as programmed cell death, or necrosis, defined as unregulated cell death. Research has since shown that the dichotomy is far less distinct than once thought, and there is still debate regarding the actual number of death modes and their categorization.[1] Study of modalities of cell death has progressed to the point where it is now understood that necrosis is subject to genetic and chemical manipulation as a regulated process under certain circumstances.[2,3] As new forms of regulated cell death are characterized, it is clear that some forms have characteristics that challenge the long-held dogma regarding the distinguishing features once thought unique to apoptosis.

For cells undergoing apoptosis, exposure of phosphatidyl serine (PS) on the outer plasma membrane serves as a signal to phagocytes, marking the cell for efferocytosis. Long considered a feature only of apoptotic cells, PS exposure has recently been reported in non-apoptotic forms of regulated cell death as well, such as necroptosis[3], a programmed form of necrosis. Indeed, much remains to be understood regarding different modes of cell death, and some notions which have been firmly engrained in cell death research may still be found to have more nuance than was once thought and may uncover new possibilities for cancer treatment.

1. Liu, X., Yang, W., Guan, Z. et al. There are only four basic modes of cell death, although there are many ad-hoc variants adapted to different situations. *Cell Biosci*. 2018; 8, 6
2. Shlomovitz, I., Speir, M. & Gerlic, M. Flipping the dogma – phosphatidylserine in non-apoptotic cell death. *Cell Commun Signal*. 2019; 17:139
3. Linkermann, A., & Green, D. R. Necroptosis. *New Eng Jrnl Med*. 2014; 370(5): 455-465.

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IMAGING CELL DEATH TO EVALUATE DRUG EFFICACY IN CANCER

As we deepen our understanding of the underlying mechanisms of different modes of cell death, finding ways to exploit these pathways with novel cancer therapies is a valid pursuit.

In vivo studies using IVISense Annexin-V 750 (AV750), an NIR fluorescent probe that detects and quantifies externalized phosphatidyl serine (PS) as a marker of cell death, to evaluate cancer drug treatment results may employ different imaging approaches. With some tumor models, it may be desirable to assess AV750 results in several different ways and at different time points to gain a more thorough understanding that may not be fully conveyed by absolute signal alone or may be confounded by imaging too early or too late.

Depending on the imaging time point post treatment and on the drug, treatment effects could present as a very dynamic process with potentially 25% of animals exhibiting increasing tumor AV750 signal after a single cytotoxic drug treatment, which may increase to ~75-100% of animals after a second or third dose. Means of assessment may include:

- Imaging general diffuse signal across the tumor
- Imaging tumor collapse with bright, focal apoptotic/necrotic regions
- Imaging high signal across the tumor

Though all of these cell death readouts are efficacious, they may quantitatively differ, therefore it is essential to understand the operant biology at the time of imaging and to maximize time-points accordingly.

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PHOSPHATIDYL SERINE

Phosphatidyl serine (PS) is an anionic phospholipid that, under homeostatic conditions, is sequestered by cells to the inner leaflet of their plasma membrane. During the induction of some forms of cell death, PS becomes externalized on the outer leaflet of the cell lipid bilayer where it functions as a signal for engulfment by phagocytic cells.

Although cell surface exposure of PS remains to be determined in all known forms of cell death (of which some estimate there are upwards of 34 proposed cell death modes[1]), it has been documented in several programmed forms, specifically in apoptosis, ferroptosis, and necroptosis.[2] When occurring in apoptosis, the immunological result of PS exposure is the release of immune dampening signals that avert an auto-immune response in normal tissues. This immune dampening ability can be exploited by cancer cells, where PS signaling may be highly dysregulated in the tumor microenvironment.[3]

In other forms of cell death, such as necrosis, proinflammatory proteins and compounds are often involved, resulting in a cascade of inflammation and tissue damage. Currently, our knowledge regarding the clearance of cells undergoing different types of cell death as well as how these cell deaths are decoded by the immune system remains limited.[2] However, it is reasonable to suggest that targeting PS activity could augment anti-cancer immune therapy, and in fact, multiple strategies have been and are being developed.[3] IVISense Annexin-V fluorescent probe can be used for non-invasive PS exposure research and in vivo evaluation of potential anticancer therapies.

VIEW IMAGE ▶

1. Liu, X., Yang, W., Guan, Z. et al. There are only four basic modes of cell death, although there are many ad-hoc variants adapted to different situations. *Cell Biosci*. 2018; 8, 6
2. Klöditz, K., Fadeel, B. Three cell deaths and a funeral: macrophage clearance of cells undergoing distinct modes of cell death. *Cell Death Discovery*. 2019; 65.
3. Dayoub, A.S., Brekken, R.A. TIMs, TAMs, and PS- antibody targeting: implications for cancer immunotherapy. *Cell Commun Signal*. 2020; 18, 29.

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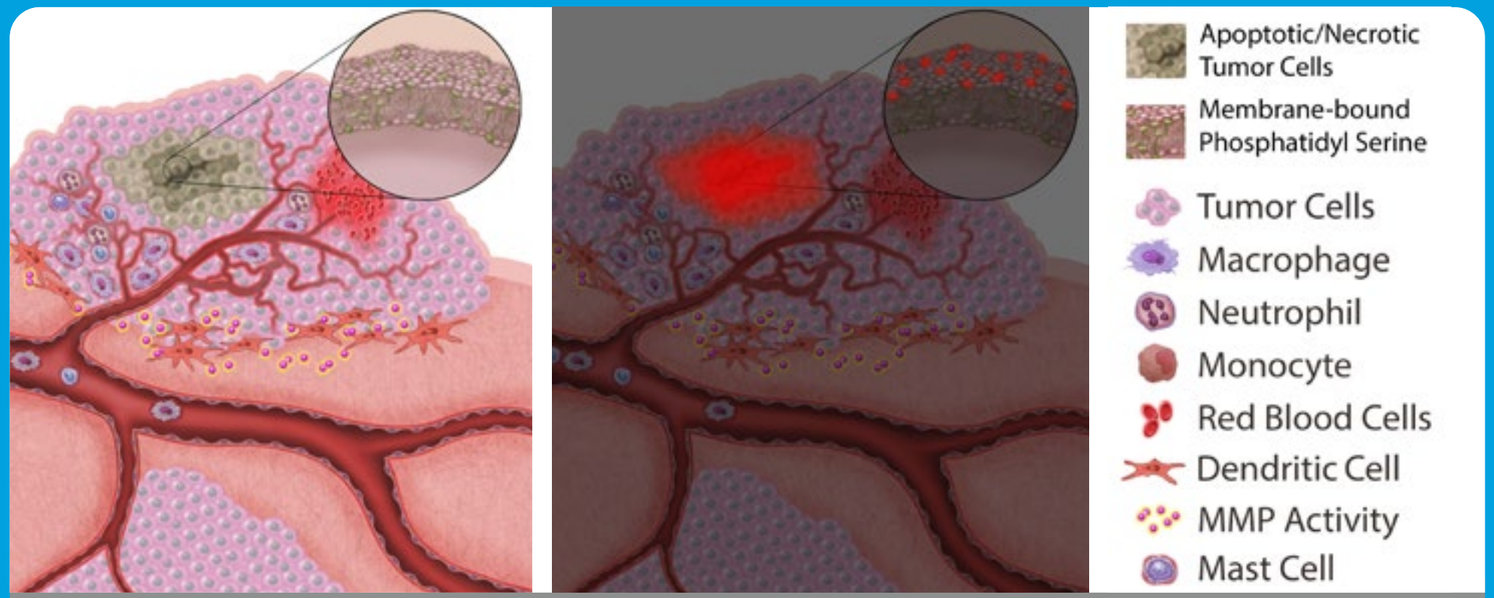
PHOSPHATIDYL SERINE



Normally confined to the inner leaflet of the cellular membrane, phosphatidyl serine (PS) distribution is disrupted and becomes exposed on the surface of cells when programmed cell death causes the membrane to become highly disordered. IVISense Annexin-V 750 fluorescent imaging of exposed PS is represented on the right.

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CONCURRENTLY TRACKED BIOMARKERS AS EARLY INDICATORS OF DRUG-INDUCED TISSUE INJURY

Though effective in treating many types of cancer, chemotherapy can produce severe side effects such as suppression of immune function and damage to heart muscle, gastrointestinal tract, and liver. If extensive enough, tissue injury can be a major reason for late-stage termination of drug discovery research projects. Therefore, it is becoming more important to integrate safety/toxicology assessments earlier in the drug development process.

There are a variety of traditional serum markers tailored mechanistically to specific tissues, however there are no current noninvasive assessment tools capable of looking broadly at *in situ* biological changes, such as cell death, inflammation, and vascular and metabolic changes, in target and non-target tissues as indicators of chemical insult. To address this need, one study found that a cocktail of *in vivo* imaging probes was a powerful tool for cancer drug efficacy/tox screening. In this study, HT-29-bearing nude mice were given a single dose of 5-Fluorouracil (5-FU) at low and high doses. Mice were tomographically imaged using a cocktail of IVISense probes: Annexin-V 750 / MMP 750 / Transferrin Receptor 750 (AMT 750), combined with IVISense Vascular 680. The very short treatment regimen showed no apparent effects on tumor mass but increasing signal from both Vascular 680 and AMT 750 was detected in heart, liver, and lungs, with the heart showing the most dramatic increase (>20-fold). The acute nature of the response, and the absence of histologic inflammation, suggested that AV 750 was detecting tissue apoptotic changes. The reversible nature of the biological changes at 24h suggested that this multi-probe approach could be a sensitive imaging strategy for detecting early tissue toxicity.

For a more complete characterization of drug efficacy/toxicity, multiple biomarkers can be tracked concurrently. By quantifying and comparing changes in the levels of exposed phosphatidyl serine, matrix metalloproteinase (MMP), transferrin receptor expression, and changes in vascularity, researchers were able to detect early tissue biological changes resulting from injury. This may provide a robust approach to the simultaneous assessment of drug-induced efficacy and collective tissue injury in mice, allowing earlier and more efficient therapeutic screening in drug discovery.

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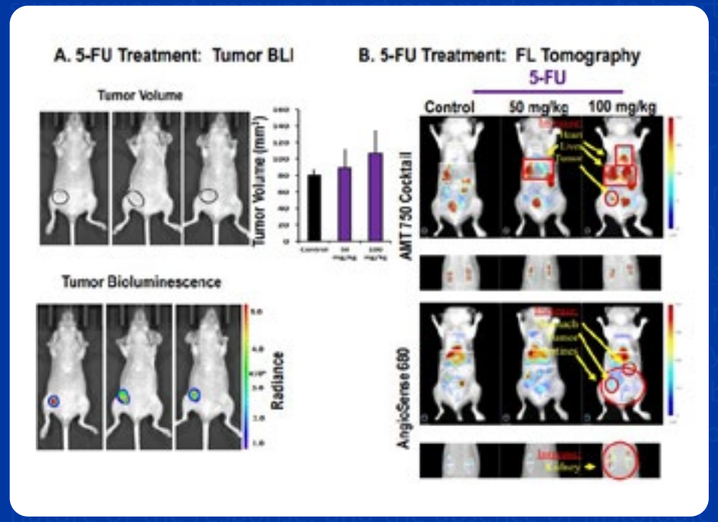
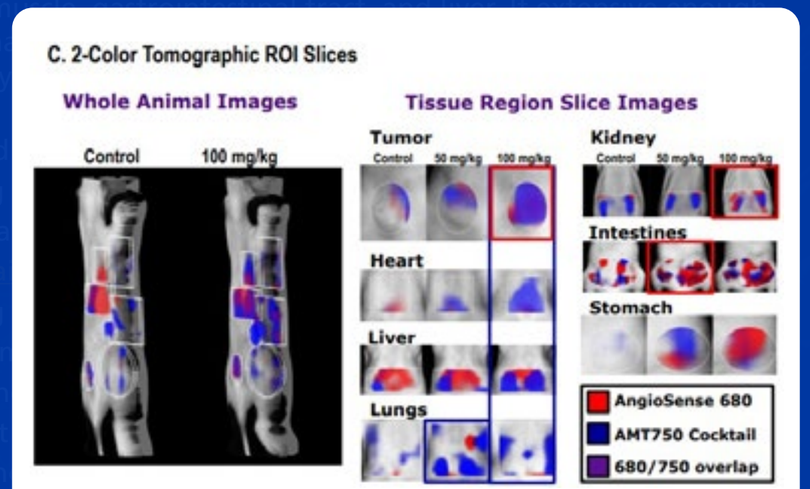
COMPLIMENTARY BIOMARKERS AS INDICATORS OF DRUG-INDUCED TISSUE INJURY[1]





HT-29 tumor bearing mice were treated with PBS, 50 mg/kg, or 100 mg/kd of 5-fluorouracil, and 2h later injected with fluorescence imaging agents. (A) Mice were injected with luciferin 12-15 min prior to imaging (at 24h) for tumor bioluminescence (IVIS® SpectrumCT). (B) AMT 750 / IVISense Vascular 680 (AngioSense 680) fluorescent signal distribution was assessed using fluorescence tomography (FMT® 4000). (C) 2-color representations of imaging datasets were rendered using 1 voxel slices through the fluorescence tomographic datasets of indicated tissue regions. Red outlined images indicate tissues showing obvious changes in signal intensity relative to controls.

1. Vasquez, Kristine O., Peterson, Jeffrey D. Combined efficacy and toxicity imaging following acute 5-FU treatment of HT-29 tumor xenografts. *Poster* (WMIC)

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CASE STUDY: VALIDATING A FOCUSED ULTRASOUND ANTI-CANCER TREATMENT BY IMAGING CELL DEATH[1]

Triggerable drug delivery systems allow for precisely placed and highly localized anti-cancer treatment with the potential advantage of lowering the systemically administered chemotherapeutic dose. In this study, researchers prepared novel MR-imageable thermosensitive liposomes (iTSL) with triggerable release properties that encapsulated doxorubicin (DOX). The resulting iTSL-DOX allowed for focused ultrasound (FUS)-mediated spatiotemporal tumor drug delivery in tumor-bearing mice with direct clinical relevance. The affects of the treatment on cancer cell viability were verified by monitoring the tumors *in vivo* with IVISense Annexin-V 750 (Annexin Vivo 750).

Using an MDA-MB-231 murine model, researchers designed a study that assessed iTSL-DOX + FUS treatment in two groups of animals, each bearing two tumors (one on each side). In one group of double-tumor mice, no iTSL-DOX was administered. The left side tumor received no FUS while the right side was treated with FUS. In the second group, mice recieved iTSL-DOX, while the left and right tumors were respectively not treated or treated with FUS in the same way as the first group.

48 hours after the first group recieved treatment with iTSL-DOX + FUS, the mice were injected with IVISense Annexin-V 750. The second group were sacrificed at the same time point with tumors excised and stained. IVISense Annexin-V 750 signal was stronger in the FUS-treated tumor to a large effect compared to the FUS-untreated tumor, indicating that the combination of iTSL-DOX and FUS enhanced tumor cell apoptosis, and histology confirmed these results, showing necrotic areas induced by the treatment. Noninvasive *in vivo* imaging with IVISense Annexin-V was able to detect iTSL-DOX + FUS cell death localized in the tumor, suggesting that the drug was released only after FUS, and that the high local dose was efficient in killing tumor cells without systemic toxicity, thereby increasing chemotherapies' therapeutic index.

See figures

1. Amrahli M, Centelles M, Cressey P, Prusevicius M, Gedroyc W, Xu XY, So PW, Wright M, Thanou M. MR-labelled liposomes and focused ultrasound for spatiotemporally controlled drug release in triple negative breast cancers in mice. *Nanotheranostics*. 2021; 5(2):125-142.

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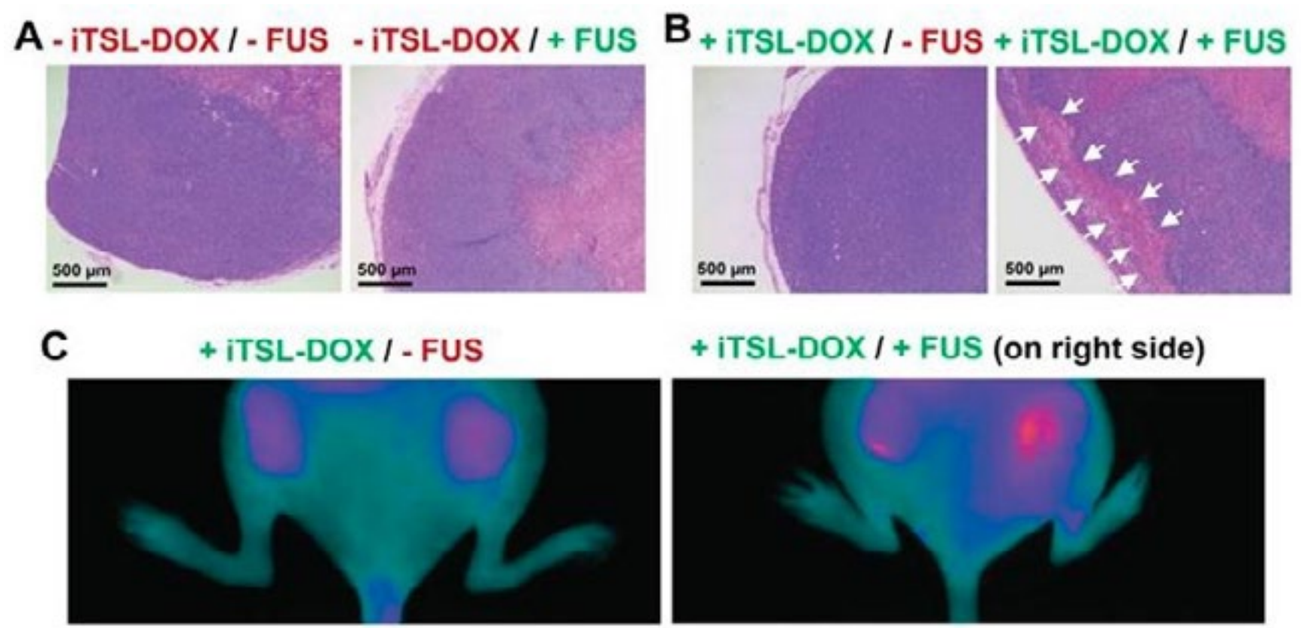

Assessing necrosis and apoptosis 48 h after treatment. (A) Histology (H & E sections), tumors were excised 48 h after the treatment. Slices (4 µm) of MDA-MB-231 tumors untreated and (B) treated with iTSL-DOX and focused ultrasound (FUS) were stained with haematoxylin and eosin. The necrotic areas induced by the treatment are indicated by the arrows. Large necrotic areas could only be found when the combination of iTSL and hyperthermia was applied; (C) Apoptosis monitoring in living mice two days post treatment using IVISense Annexin-V 750, with images recorded 3-4 h after the administration. Significant increases in apoptosis were apparent on the right side where FUS had been applied.

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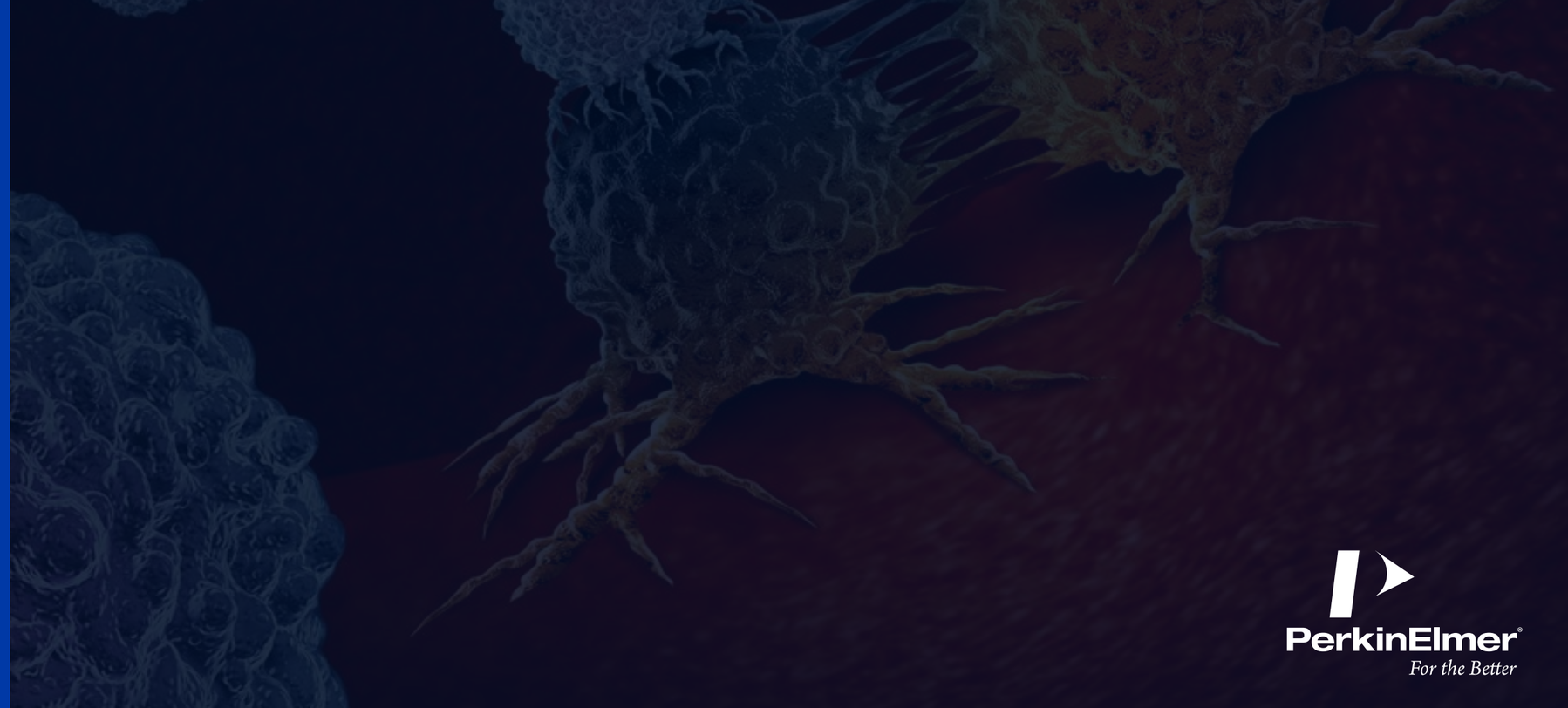
BY CHARACTERIZATION | **BIOPROFILING**

Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25 g)	Sold As (Unit Size)	Optimal Imaging Time (h)
⌖ IVISense Annexin-V 750	35,000	Phosphatidyl serine exposure on cells	Targeting of membrane phosphatidyl serine exposed on cells undergoing apoptosis/necrosis/other forms of cell death.	100 µL	1 mL	2

⌖ Targeted probes actively target and bind to distinct biomarkers with highly specific targeting to key biological mechanisms.



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Toxicology Panel
Set of Fluorescent Probes Bundled by Application

IVISense Probe	MW (g/mol)	Specificity	In Vivo Biology Use	Mouse Dose (25 g)	Amount (Unit Size)	Optimal Imaging Time (h)	Probe Clearance
Vascular 680	~70,000	NA	Vascular probe. Leaks into sites of vascular damage or inflammation.	2 nmol	24 nmol	24	6-7 d
Transferrin Receptor 750	106,000	Transferrin receptor	Detects upregulation of transferrin receptors on tumor cells. Marker for iron metabolism in liver.	2 nmol	24 nmol	24 (6-24)	96 h
MMP 750 FAST	43,000	Matrix Metalloprotease	Cleaved by multiple metallo-proteinases. Secreted markers of stellate cells, Kupffer cells, macrophages, and neutrophils.	4 nmol	48 nmol	24 (12-24)	6-7 d
Annexin-V 750	35,000	Phosphatidyl serine exposure on cells	Cell death/apoptosis probe. Marker on inflammatory cells; metabolic marker on tumor cells.	100 µL	1 mL	2	3 d
Renin 680 FAST	43,000	Renin protease activity	Renin activatable, marker of tissue renin-angiotensin system activity.	2 nmol	24 nmol	24 (12-24)	4 d

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BEST PRACTICES IN FLUORESCENCE MOLECULAR IMAGING

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APPROACHES TO FLUORESCENCE MOLECULAR IMAGING (ILLUSTRATION)

VIEW ▶

PROBE REFERENCE TABLES

Characterization of the full portfolio of IVISense probes:
Activatable, Targeted, Vascular

VIEW ▶

REFERENCES

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Fluorescence molecular imaging is the visualization of cellular and biological function *in vivo* to gain deeper insights into disease processes and treatment effects.

One key aspect of successful *in vivo* imaging is using carefully selected, validated probes. Importantly, probes with high specificity and biologically appropriate characteristics, such as clearance rate and route of metabolism, will enable you to gain unique and meaningful biological information from your fluorescent imaging studies.

PerkinElmer's fluorescent imaging probes are developed through an extensive R&D process, designed to incorporate drug-like biodistribution properties, and validated for optimal target delivery and performance. By considering both the biology of interest and the probe characteristics, you can design imaging studies that yield better quantification of early biological changes, and allow you to "see" biological activity in a living animal.

PerkinElmer has a variety of NIR fluorescent imaging probes that can be used to:

- Study complex biology
- Multiplex up to 4 imaging probes
- Perform deep-tissue imaging
- Combine with bioluminescent microCT, and PET imaging for multimodal studies

DESIGNING AN EFFECTIVE STUDY

Fluorescence molecular imaging approaches can differ depending on your ultimate goal. The following are key steps to designing a fluorescent imaging study:

1. Set Clear Goals for Your Study, and Identify Which Approach Will Maximize Your Research

- Give careful consideration to which imaging approach will provide the most appropriate benefits (see Approaches to Fluorescence Molecular Imaging – Illustration).

For example, is your primary goal to explore early disease, or late disease? Waiting until the end of the study to understand the limited biology available at that time can miss a lot of biology that has already happened. With the proper imaging approach, it's possible to draw useful

distinctions between tissues when looking at critical early timepoints, where tissues have similar status and only subtle but critical biological differences. Later imaging sees only gross overt physiological changes from disease, with gross overt biological differences. Thus, at later points, it becomes harder to make meaningful comparisons, given the very different biological landscapes being comparing.

2. Know Your Animal Model's Biology Up Front (Cellular Players, Kinetics)

- The timing and/or kinetics of biology is important. If you are imaging cells that don't arrive on the scene until a certain time, then it is important to plan your probe injection and the imaging window to coincide with when the biology is operant.

3. Identify Optimal Probe Objectives

- What do you need your probe to be able to do? Examples of probe objectives could be to bind a specific cell surface receptor, or fluoresce only in the presence of a specific protease or biomarker.
- If antibodies are how you plan to perform your imaging, be cautious about using intact antibodies as probes.

4. Understand Probe Performance for Alignment with Your Model

- Consider probe characteristics such as route of metabolism, time frame from injection to imaging, or tissue washout time (Refer to Technical Datasheets on website).
- Test probes to identify best imaging strategies, timepoints, and analysis approaches. Depending on your imaging strategy, choosing probes to detect disease markers may yield early biological information to characterize disease onset and progression, whereas probes for detecting treatment markers may provide data predictive of non-imaging terminal readouts.
- Many of our probes are designed to be universally applicable for most diseases, which usually manifest some component of inflammation, vascular damage, metabolic changes, or cell death – aspects of disease that our probes are acutely capable of detecting. There may not be a "tailored" probe to your target biology, however other more downstream probes may be effective.

5. Choose Proper Imaging Controls

- Appropriate study design should include both positive control and negative control (i.e. un-diseased) mice injected with probe(s).

6. Confirm Results *In Vivo* with Careful *In Situ* And/Or *Ex Vivo* Tissue Corroboration

- Use fluorescent probes suitable for *ex vivo* imaging and even frozen sections for microscopy with appropriate excitation/emission spectra.

7. Validate Imaging Results Against "Gold Standard" Non-Imaging Metrics

- Proper imaging results will correlate with or sometimes surpass current "gold standard" readouts, such as histology.

GENERATING A PRECLINICAL IMAGING MODEL

In new model development, it is essential to validate your imaging results with non-imaging *ex vivo* observations/quantifications as well as with established "gold standard" readouts.

IMAGING WITH INTACT ANTIBODIES

Though easy to label and highly target specific, intact antibodies have several disadvantages, including:

- A very long circulating half-life
- Poor tissue penetration
- A tendency for the Fc receptor to bind phagocytic cells

For these reasons, accumulation of intact antibodies in off-target biology is possible, which raises concerns regarding misinterpretation of experimental results. Ab fragments are a better choice, and can be labeled with PerkinElmer's IVISense® Fluorescent Dyes, designed to have minimal interaction with biological tissue.

When adapting an animal model for *in vivo* fluorescent imaging, it is important to know as much as possible about the biology and its kinetics in your model. Important factors include:

- Time to first biological changes; time to overt clinical disease
- If using a therapeutic treatment, do you understand the biological changes that will be induced?
  - For example, in cancer, cytotoxic drug activity may be detected by imaging cell death such as IVISense™ Annexin-V, whereas an anti-angiogenic efficacy may be best detected with a vascular probe such as IVISense Vascular.
- Optimal probe(s) injection time
- Deep tissue or superficial tissue imaging; 2D vs 3D (or *ex vivo*) imaging
- The anatomical region to be imaged and its proximity to difficult areas, such as liver, kidney, or bladder, where some probes will metabolize. This can cause high background signal, and should be a consideration in probe selection.
- The cellular players in the model (i.e. neutrophils, macrophages, lymphocytes, tumor cells, bacteria, hepatocytes, etc.)
- Some probes will be obvious choices based on the cells involved (e.g. IVISense Pan Cathepsin or IVISense MMP for inflammatory cells)

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PROPER IMAGING TECHNIQUE AND ANALYSIS CONSIDERATIONS

In conjunction with properly selected, validated probes, an emphasis on (i) effective study design, (ii) understanding your imaging model, and, (iii) careful imaging technique as detailed in the next section, you can maximize your *in vivo* biological readouts to detect early indicators of standard endpoints, and realize more meaningful research results.

Best Practice Techniques for *In Vivo* Imaging:

- Depilation
- Consistent Animal Positioning
- Injection Method
- Proper Analysis

DEPILATION

To minimize light scattering and absorption in fluorescence imaging, hair should be removed from the appropriate body region of all mice. Figure 1 demonstrates the significance of proper depilation. Hair removal can be performed under proper depilation.

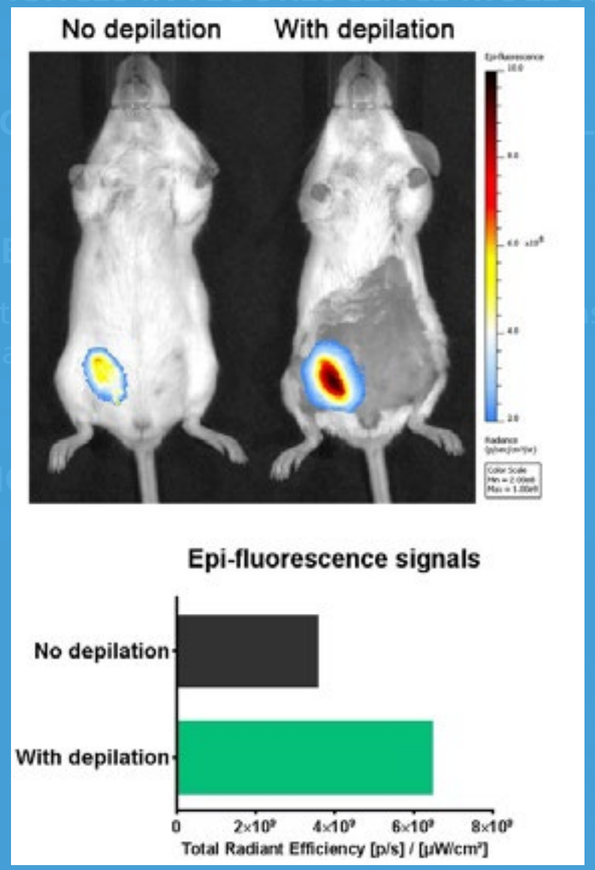

Figure 1. The image above demonstrates the impact of no depilation vs. depilation on epi-fluorescence signal.

Hair removal can be performed under injectable or inhaled anesthesia. Care should be taken to remove hair from an area larger than just the region of focus to assure that target and surrounding background fluorescence can be captured. For tomographic imaging, hair must be removed from front, sides, and back for the region of focus.

Once animals are properly anesthetized, apply depilatory cream (Nair™ lotion, Church and Dwight Co., Inc., Princeton, NJ) thickly on hair over the imaging region of each mouse. Rinse off thoroughly with warm water and reapply until all hair has been removed.

IMPORTANT: Rinsing must be done carefully and thoroughly to minimize any introduction of skin lesions that can cause imaging artifacts. Take care to ensure that the mouse does not swallow or breathe in any water during rinsing. Once mouse has been depilated fully, move to a heating pad or to its original cage.

CONSISTENT ANIMAL POSITIONING

Animal positioning can influence the epi-fluorescence signal, as demonstrated in Figure 2. It is important to ensure that proper positioning based on tumor location is consistently used.

INJECTION METHOD

- For most of the probes, intraperitoneal (IP) or subcutaneous (SC) injection will either not work at all or will be extremely variable with high injection site signal. All probes must be injected systemically either through the retro-orbital plexus or the tail vein.

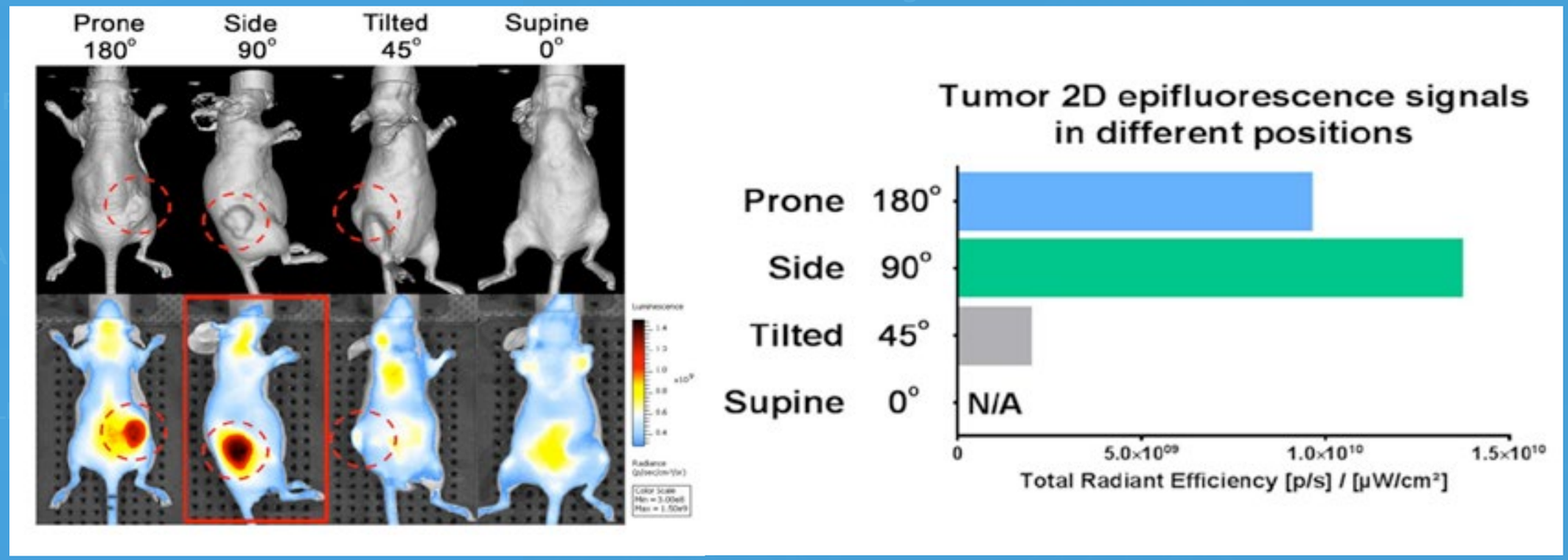

Figure 2. The images to the right demonstrate the impact of animal positioning on epi-fluorescence signal.

PROPER ANALYSIS

When analyzing fluorescence imaging data (even without taking fluorescent background into account) the results are valid and will provide robust and statistically significant data. However, although fluorescent imaging probes can provide excellent targeting of disease biology, they also distribute widely throughout the body and can vary in routes of clearance and kinetics of background Interference. In some cases (depending on the intensity of the signal at the target) more that 50% of the signal in the target region could be attributed to background contribution. This means that some sort of objective and consistent approach for correcting data for background contribution is needed for proper interpretation.

This is particularly important when making other calculations, such as % inhibition, background, or ratios of two different imaging probes.

Consider Figure 3 below, showing hypothetical datasets in which there are untreated animals and treated animals but the examples differ only in background levels. Assuming there is no background yields a calculation of 44% inhibition (Example 1), however Examples 2 and 3 show the impact of a modest background and a high background, with percent inhibition raising to 57% and 80% respectively. Taking background into account in your analysis will provide you with higher precision and improved data interpretation.

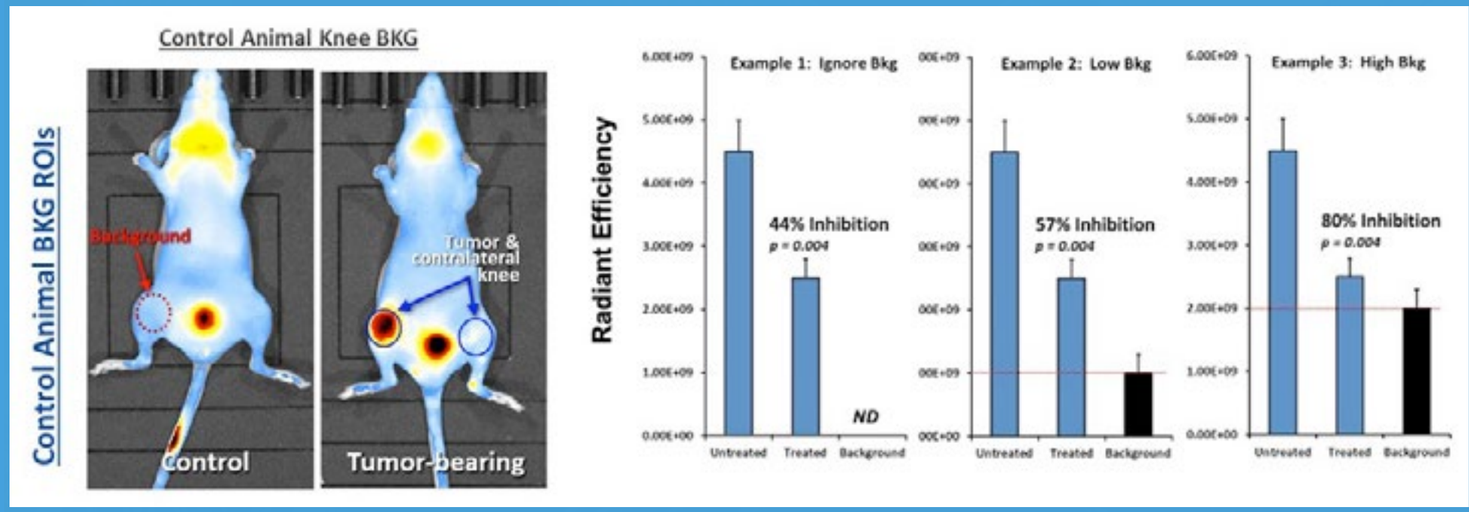

Figure 3. Proper image analysis can greatly impact interpretation of study results.

PROTOCOL FOR MONOPLEX, MULTIPLEX, AND LONGITUDINAL IMAGING WITH IVISENSE FLUORESCENT PROBES

Study Preparation

1. Two weeks before the imaging study, switch mice to low fluorescence chow. Regular mouse chow contains chlorophyll that auto fluoresces around 700 nm which can interfere with imaging.
2. On the study day, it is essential to prepare ahead of time for optimal results. Group and number the mice to be injected and imaged. Remember that appropriate study design should include both positive control and negative control (i.e. un-diseased) mice injected with probe(s).
3. Mouse hair removal is essential for sensitive, high quality fluorescence imaging. Either genetically hairless mice (SKH-1E) or normal, haired mice (BALB/c, C57BL/6, etc.) with depilation must be used for optimal fluorescence tomographic imaging. Follow the depilation procedure above for haired mice.
4. Establish the readiness of the imaging system by checking the anesthesia chamber and connections to the system. Activate the anesthesia, setting evaporator to the appropriate settings for your particular set-up.
5. Make sure you know ahead of time the proper positioning of the mouse you will use to facilitate acquisition of the best quality data for your particular animal model.

Single Probe Imaging

1. Prepare the imaging probe according to included instructions. Note that systemic injection is required, either through the retro-orbital plexus or the tail vein.
2. Place a heating pad beneath the anesthesia induction chamber to keep the body temperature of the mice constant. Be careful not to overheat. Anesthetize the first mouse by placing it in a gas anesthesia induction chamber.
3. Remove the mouse from the induction chamber when it appears completely anesthetized, and confirm the depth of anesthesia through unresponsiveness to toe pinch.
4. Inject the appropriate volume of probe (100 - 150 µL, as per each probe's specific instructions) via the retro-orbital plexus (or tail vein) of the anesthetized mouse. Record the injection time.
5. Return the mouse to the cage for recovery and go to the next mouse for injection.
6. Repeat steps 1 - 6 until all mice are injected.
7. Imaging is performed at the suggested time(s) using a single excitation/emission filter pair optimal for the wavelength of the probe wavelength of the probe to be imaged (see table). Anesthetize mice using inhaled anesthesia and place them carefully in the appropriate orientation in the imaging system. Multiple images can be acquired with little or no concern for photobleaching of the probes.

Two Probe Imaging

1. Prepare the first imaging probe as described in that probe's instructions. Note that systemic injection is required, either through the retro-orbital plexus or the tail vein.

   To minimize injection volume, either use the Probe 1 solution to solubilize Probe 2 or make each probe at half-volumes for mixing. (Note: Bear in mind that three of the probes come in 10X liquid form: IVISense Annexin-V 750, MMP 680, and Pan Cathpsin 680). Multiple specific strategies for preparation are possible, but it is ideal to keep mouse injection volumes under 250 µL.
2. Prepare and inject mice as described above in Single Probe Imaging.
3. Imaging should be performed as described in step 7 above, but for both 680 nm and 750 nm using the appropriate excitation and emission filter pairs. Correct times for acquisition should be noted; although most of the probes are optimal for 24 h imaging, some have flexibility to be imaged earlier, while others should only be imaged earlier. For example, IVISense Annexin-V 750 imaging is optimal for most applications at 2 hours, whereas Pan Cathepsin 680 is optimal at 24 hours. You can either image both wavelengths at both 2 hours and 24 hours, or you can image 750 nm at 2 hours and 680 nm at 24 hours.
4. Multiple repeat acquisitions can be performed with little or no concern for photobleaching of the probes.

Longitudinal Imaging

1. PerkinElmer probes are well characterized with respect to tissue clearance kinetics, providing guidance for longitudinal imaging strategies. Depending on the probe, reinjection generally can be performed three to seven days following the first image acquisition.
2. Imaging bone turnover with IVISense Osteo 680 requires a different strategy due to the very long tissue clearance kinetics. Secondary imaging time points must be performed using a pre-imaging strategy; briefly, mice should be imaged immediately prior to each additional probe injection to allow subtraction correction of additional imaging datasets.

APPROACHES TO FLUORESCENCE MOLECULAR IMAGING

IMAGING STRATEGY, TIME POINTS, AND READOUTS

		Study Goal	Disease Progression (EARLY – LATE)	Treatment Progression (EARLY – LATE)	Benefit
Detect Disease Markers	GOOD	Diagnostic Imaging	Detect or confirm presence of disease or condition of interest using diagnostic marker.		Simple, fast diagnostic screening
	BETTER	Predictive Imaging/ Bioprofiling	Detect predictive marker or bioprofiling markers.* Key to this strategy is incorporating earlier imaging time points, prior to overt biological changes, to capture important biology.		Focus on early detection of key biology
		Disease Onset Bioprofiing	Characterize multiple* biomarkers by longitudinal biology imaging over course of disease.		Focus on characterizing and understanding biology progression
Detect Treatment Markers	GOOD	Surogate Endpoints		Correlate with non-imaging terminal readouts by imaging at end of dosing protocol.	Simple, fast screening
	BETTER	Biological Endpoints/ Therapeutic Bioprofiling		Predictive of non-imaging terminal readouts; may use multiple* biomarkers for more complete data.	Improved readouts in alignment with underlying histophathology
		Predictive Efficacy		Detect pharmocologic response shortly after treatment,prior to surrogate or clinical endpoints.	Detection of therapeutic efficacy with as few as 1-3 drug doses

*Multiplex Imaging (See “Two Probe Imaging” in Best Practices in Fluorescence Molecular Imaging for a detailed protocol.)

PROBE REFERENCE TABLE— ACTIVATABLE PROBES

NOTE: Blood t1/2 in hours or fractions of hours

Cat #	IVISense Probe	MW (g/mol)	Specificity	*In Vivo* Biology Use	Mouse Dose (25g)	Rat Dose (250g)	Route of Injection	Blood t1/2 (h)	Tissue t1/2 (h)	Optimal Imaging Time (h)*	Optimal Reinjection Time – Complete Clearance	Route of Metabolism/ Background Tissue(s)
NEV11112	**Cat B 680 FAST**	33,000	Cathepsin B	Selectively cleaved by cathepsin B proteinases upregulated in tumors and inflammatory cells.	2 nmol; (4 nmol atherosclerosis)	6-20 nmol	IV	1	36	6-24	3 d	Salivary glands > liver, kidneys
NEV11098	**Cat B 750 FAST**	23,000			4 nmol; (8 nmol atherosclerosis)	12 nmol	IV	1	36	6-24	3 d	Salivary glands > liver, kidneys
NEV11000	**Cat K 680 FAST**	8,500	Cathepsin K	Selectively cleaved by cathepsin K proteinases upregulated in osteoclasts and tumor-associated macrophages.	2 nmol	6-20 nmol	IV	1/2	36	6-24	3 d	Kidney > liver
NEV11100	**MMP 645 FAST**	43,000	Matrix Metallo-protease	Cleaved by multiple metalloproteinases associated with many disease areas including cancer and inflammation.	4 nmol	12-40 nmol	IV	5	72	24 (6-24)	6-7 d	Liver > kidneys
NEV10126	**MMP 680**	~450,000			2 nmol	6-20 nmol	IV	5	72	24 (12-24)	6-7 d	Liver
NEV10168	**MMP 750 FAST**	43,000			2 nmol	6-20 nmol	IV	5	72	24 (24-36)	6-7 d	Liver > kidneys
NEV11169	**Neutrophil Elastase 680 FAST**	43,000	Neutrophil Elastase	Selectively cleaved by elastase produced by activated neutrophils during acute inflammation.	4 nmol	12-40 nmol	IV	3	12	3-6	2 d	Bladder > liver, intestines
NEV10003	**Pan Cathepsin 680**	~400,000	Cathepsin proteases	Pan-cathepsin activatable agent that detects abnormal upregulation of cathepsin secretion associated with cancer and inflammation.	2 nmol	6-20 nmol	IV	12	72	24 (24-48)	6-7 d	Liver
NEV10001EX	**Pan Cathepsin 750**	~450,000			2 nmol	6-20 nmol	IV	5	72	24	6-7 d	Low liver, intestine
NEV11171	**Pan Cathepsin 750 FAST**	22,500			4 nmol	12-40 nmol	IV	1	36	6-24	3 d	Low liver, bladder
NEV11079	**Renin 680 FAST**	43,000	Renin Protease	Activated by normal kidney renin and upregulated renin-angiotensin system (RAS) activity.	2 nmol	6-20 nmol	IV	7	48	24 (12-24)	4 d	Kidney, lung, liver

Activatable probes are optically silent upon injection and become highly fluorescent following protease-mediated activation.

* Animal injection times should be staggered to meet imaging timepoint(s) +/- 0.5 h.

PROBE REFERENCE TABLE— TARGETED PROBES

NOTE: Blood t1/2 in minutes

Cat #	IVISense Probe	MW (g/mol)	Specificity	*In Vivo* Biology Use	Mouse Dose (25g)	Rat Dose (250g)	Route of Injection	Blood t1/2 (h)	Tissue t1/2 (h)	Optimal Imaging Time (h)*	Optimal Reinjection Time – Complete Clearance	Route of Metabolism/ Background Tissue(s)
NEV11053	**Annexin-V 750**	35,000	Phosphatidyl serine exposure on cells	Targeting of membrane phosphatidyl serine exposed on cells undergoing apoptosis/necrosis/ other forms of cell death.	100 µL	300 µL	IV	15	14	2	3 d	Kidneys (high), liver
NEV10090	**Bombesin Receptor 680**	24,000	Bombesin receptor	Detects upregulation of bombesin receptors on tumor cells associated with increased tumor proliferation.	2 nmol	6-20 nmol	IV	90	96	24	6-7 d	Pancreas, kidneys
NEV10040	**Folate Receptor 680**	1,606	Folate receptor	Detection of folate receptor protein upregulation for monitoring tumor growth and metabolism.	2 nmol	6-20 nmol	IV	5	>168	6 (6-24)	6-7 d	Kidneys
NEV11070	**Hypoxia CA IX 680**	1,500	Carbonic Anhydrase IX	Detection of carbonic anhydrase 9 (CA IX) protein expressed in many tumor cell lines undergoing hypoxia and acidosis.	2 nmol	6-20 nmol	IV	2	12	24	6-7 d	Kidneys
NEV10640	**Integrin Receptor 645**	1,250	αVβ3 Integrin	Detection of integrin αVβ3 expression in tumors and neovasculature.	2 nmol	6-20 nmol	IV (IP)	10	48	6-24	6-7 d	Bladder, kidneys
NEV10645	**Integrin Receptor 680**	1,432			2 nmol	6-20 nmol	IV (IP)	10	24	24	14 d	Kidneys, intestines
NEV10873	**Integrin Receptor 750**	1,278			2 nmol	12-40 nmol	IV (IP)	30	24	24	4-6 d	Kidneys
NEV10020EX	**Osteo 680**	1,471	Hydroxyapatite	Binds exposed hydroxyapatite (HA), a biomarker for osteoblast activity; abnormal HA levels can indicate bone damage/disease, such as in metastasis.	2 nmol	6 nmol	IV (IP)	5-10	~30 days	3-24	Preimage subtraction	Bladder
NEV10053EX	**Osteo 750**	1,101			4 nmol	12 nmol	IV (IP)	5-10	7-10 days	3-24	Preimage subtraction	Bladder
NEV11105	**Osteo 800**	1,281			2 nmol	6 nmol	IV (IP)	5-10	7-10 days	3-24	Preimage subtraction	Bladder
NEV10060	**Tomato Lectin 680**	~72,000	Endothelial glycoproteins	Vascular burden assessment through binding of glycoprotein N-actylglucosamines on the surface of vascular endothelial cells.	2 nmol	6 nmol	IV	2	>48	6	4-6 d	Overall vascular background
NEV10091	**Transferrin Receptor 750**	106,000	Transferrin receptor	Detects upregulation of transferrin receptors on tumor cells associated with tumor metabolism.	2 nmol	6-20 nmol	IV	10	24	24 (6-24)	96 h	Liver, kidney

Targeted probes actively target and bind to distinct biomarkers with highly specific targeting to key biological mechanisms.

* Animal injection times should be staggered to meet imaging timepoint(s) +/- 0.5 h.

** Some probes can be administered IP, but they may leave a residual peritoneal signal that could interfere with imaging.

PROBE REFERENCE TABLE— VASCULAR & PHYSIOLOGICAL PROBES

NOTE: Blood t1/2 in hours or fractions of hours

Cat #	IVISense Probe	MW (g/mol)	Specificity	*In Vivo* Biology Use	Mouse Dose (25g)	Rat Dose (250g)	Route of Injection	Blood t1/2 (h)	Tissue t1/2 (h)	Optimal Imaging Time (h)*	Optimal Reinjection Time – Complete Clearance	Route of Metabolism/ Background Tissue(s)
NEV10117, NEV10130	**Acute Vascular 680 (1mg, 5mg)**	1,646	NA	Small molecule fluorescent agent that can be used as a control for vascular permeability imaging.	50 µg	150-500 µg	IV	0.2	Not determined	0.5	Not determined	Bladder
NEV10118, NEV10177	**Acute Vascular 750 (1mg, 5mg)**	1,086										
NEV10116	**Edema 680**	~1,540	Passive; non-specific systemic circulation	Imaging of vascularity, perfusion, and vascular permeability. Short pharmacokinetic profile.	4 nmol	12-40 nmol	IV	1.5	5-10	3 (1-3)	96 h	Bladder
NEV11121	**Gastrointestinal 750**	~40,000	NA	Monitoring of gastric emptying/motility.	0.25 nmol	0.75-2.5 nmol	Oral gavage	NA	NA	Kinetic 5 min-3 h	3-5 h	Stomach, intestines
NEV30000	**GFR 680**	~6,000	NA	Quantitative assessment of renal Glomerular Filtration Rate (GFR), as an indicator of renal toxicity or injury.	2 nmol	6-20 nmol	IV	See protocol on website to include Optimal Reinjection Time.				Kidneys
NEV10054EX	**Vascular 680**	~70,000	Passive; non-specific systemic circulation	Imaging of vascularity, perfusion, and vascular permeability.	2 nmol	6-20 nmol	IV	7	72	24 (image vasculature for up to 4h; Tumor accumulation at 24h)	6-7 d	Low liver ling
NEV10011EX	**Vascular 750**							16				
NEV10149	**Vascular NP 680**	~4,000,000 (20-50 nm diameter)	Passive; non-specific systemic circulation	Imaging of vascularity, perfusion, and vascular permeability. Long pharmacokinetic profile.	200 µL	300-1000 µL	IV	20	>100	24	Preimage subtraction	Long term tissue accumulation
NEV10150	**Vascular NP 750**											

Vascular & Physiological Probes are a range of highly fluorescent *in vivo* imaging molecules that remain highly stable and localized in the anatomy for various periods of time to enable imaging of disease physiology, vasculature, vascular permeability, and angiogenesis.

IVISense Vascular NP 680 & 750 probes are highly fluorescent near infrared nanoparticles specifically designed for *in vivo* imaging. They remain localized in the vasculature for extended periods and enable imaging of blood vessels and angiogenesis.

* Animal injection times should be staggered to meet imaging timepoint(s) +/- 0.5 h.

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TUMOR BURDEN
- Folate
- MMP

METABOLIC ACTIVITY
- Cathepsin

ANGIOGENESIS
- Carbonic Anhydrase IX

METASTASIS
- Phosphatidylserine
- Endothelium
- αvβ3 Integrin

DRUG RESISTANCE
- Bombesin

INFLAMMATION
- Elastase
- Hydroxyapatite

CELL DEATH
- Transferrin

RESOURCES

CONTACT US

We can help you design your fluorescence experiment, choose the right probe, or interpret your data. Talk to a scientist before starting your next study, or set up a meeting for your whole team to learn more.

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