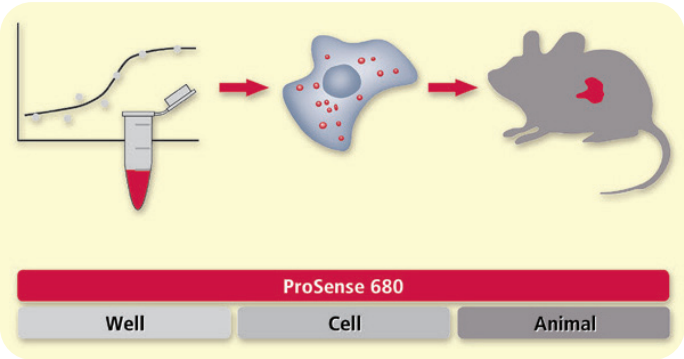
Visualization of cathepsin activity using IVISense Pan Cathepsin 680 agent from well to cell to animal.

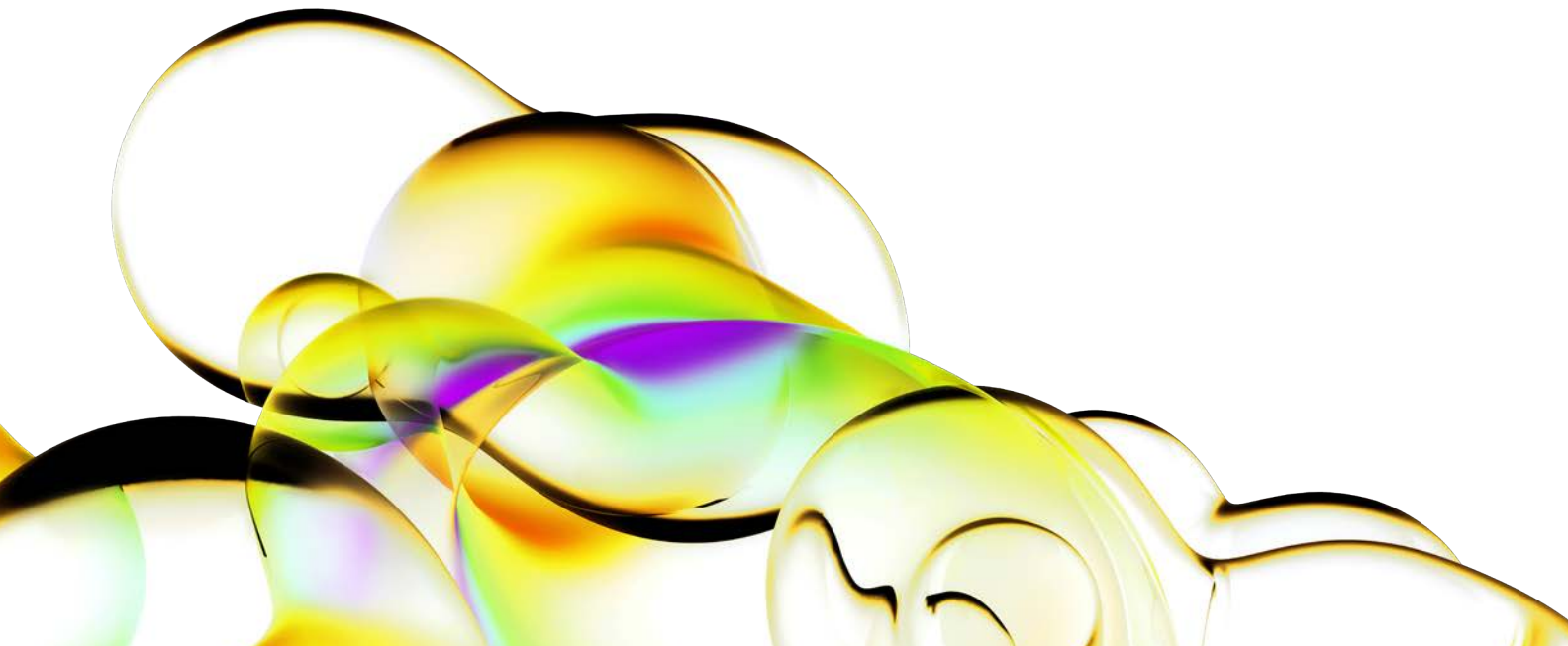
Key Features

- Biochemical cathepsin activity assay
- Visualization of cathepsin activity in a cell toxicity assay
- Pre-clinical *in vivo* cathepsin imaging of tumor progression and treatment

Introduction

Cathepsins are proteases, detected at basal levels in all living cells (Turk et. al., 2001), that are known to play an important role in several physiological processes such as lysosomal protein degradation, antigen presentation and apoptosis. However, cathepsins are also associated with pathological conditions and diseases such as cancer, osteoporosis and bronchial asthma (Turk et. al., 2001; Zavasnik-Bergant & Turk, 2006; Vasiljeva et. al., 2007). In cancer in particular, there is a significant increase in lysosomal cathepsins at the mRNA and protein level, and these proteases have shown considerable potential as biomarkers for tumor progression and metastasis. As cathepsin activity is regulated by post-translational intracellular processing (Katunuma, 2010), requiring the diverse functions of a living cell, cathepsins may also serve as biomarkers of tumor cell viability. In summary, their well-established role in tumor development, progression and metastasis (Mohamed & Sloane, 2006; Conus & Simon, 2010) makes cathepsins attractive targets for visualization and characterization of viable tumor cells.





Cathepsin-activatable agents such as the IVISense™ Pan Cathepsin 680 and IVISense Pan Cathepsin 750 Fluorescent Imaging Agents are optically silent in their intact, inactive states but become highly fluorescent upon endocytosis and protease-mediated cleavage by specific cathepsins within lysosomes. IVISense Pan Cathepsin 680 and IVISense Pan Cathepsin 750 agents (Revvity, NEV10003 and NEV11171) each consist of a pegylated poly-lysine backbone that is highly labeled with near infrared (NIR) fluorophores (Bremer et. al., 2002). In the IVISense Pan Cathepsin agents' native states, fluorophore labeling density is high enough to result in mutual energy transfer, thus quenching fluorescence (Figure 1). Unmodified lysine residues serve as cleavage sites for proteases, predominantly those of the cathepsin family that recognize lysine-lysine sites. After proteolytic cleavage of the poly-lysine backbone, the fluorophores are released from quenching, resulting in a bright fluorescent signal that can be detected *in vitro* (protease and cellular assays) and *in vivo* as an indicator of the presence of cathepsin activity. After excitation with 680 nM light, IVISense Pan Cathepsin 680 agent shows a NIR fluorescence signal (emission max. 700 nM). Light absorption and scattering, as well as tissue and cellular autofluorescence, is minimal in the near-infrared spectral region (700-900 nM), providing optimal imaging conditions in cells, tissues and small animals.

In this study we show an example of the translational use of NIR agents across the range of the drug discovery process, from biochemistry to cellular assays to *in vivo* testing, using HT-29 colorectal cancer cells and 5-Fluorouracil (5-FU) treatment.

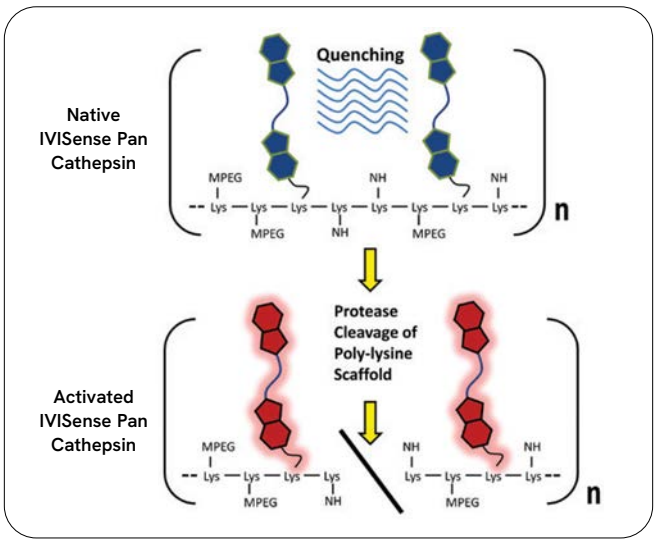


Figure 1: IVISense Pan Cathepsin imaging agent fluorescence is activated by protease mediated cleavage.

Results

Biochemical profiling of IVISense Pan Cathepsin fluorescent imaging agent

Activation of IVISense Pan Cathepsin agent (0.5 µM) was determined with a panel of relevant enzymes (0.1 µM final concentration): recombinant human cathepsin B, D, L, S (R&D Systems, Minneapolis, MN), matrix metalloprotease (MMP)-2, 9, 12, 13 (Enzo Life Sciences, Farmingdale, NY), and legumain (R&D Systems, Minneapolis, MN). Recombinant enzymes were activated, as necessary, according to the manufacturer's instructions. The assay buffer and pH conditions were optimized in previous studies for optimal cleavage of known substrates, as well as the IVISense Pan Cathepsin agent. The specific assay buffers are listed in Table 1. Reactions were carried out in a 250 µL volume and set up in 96-well plates with black sides and bottom. Reactions were monitored at various time points (0, 1, 3, 5, 24 h) and at excitation/emission wavelengths of 663/690 nM or 738/775 nM, as appropriate, using a fluorescence plate reader. The emitted fluorescence was determined by subtracting the agent-only background (i.e. in the absence of recombinant protease). The results presented in Figure 2 show the selectivity of IVISense Pan Cathepsin agent for the cysteine cathepsins B, L, and S, whereas the aspartyl cathepsin D, legumain, and a variety of MMPs were unable to cleave and activate the IVISense Pan Cathepsin agent.

Table 1: Biochemical assay conditions.

Protease	Class	Assay buffer
Cathepsin B	Cysteine protease	25 mM MES (pH 5.0)
Cathepsin D	Aspartyl protease	100 mM Formate Buffer (pH 3.3)
Cathepsin L	Cysteine protease	50 mM MES (pH 6.0), 5 mM DTT
Cathepsin S	Cysteine protease	50 mM NaOAc (pH 4.5), 5 mM DTT, 0.25 M NaCl,
MMP 2	Type IV collagenase	50 mM Tris (pH 7.5), 10 mM $CaCl_2$, 150 nM NaCl, 0.05% Brij-35
MMP 9	Gelatinase	50 mM Tris (pH 7.5), 10 mM $CaCl_2$, 150 nM NaCl, 0.05% Brij-35
MMP 12	Macrophage elastase	50 mM Tris (pH 7.5), 10 mM $CaCl_2$, 150 nM NaCl, 0.05% Brij-35
MMP 13	Type II collagenase	50 mM Tris (pH 7.5), 10 mM $CaCl_2$, 150 nM NaCl, 0.05% Brij-35
Legumain	Asparaginyl endopeptidase	50 mM MES, 250 mM NaCl, pH 5.0

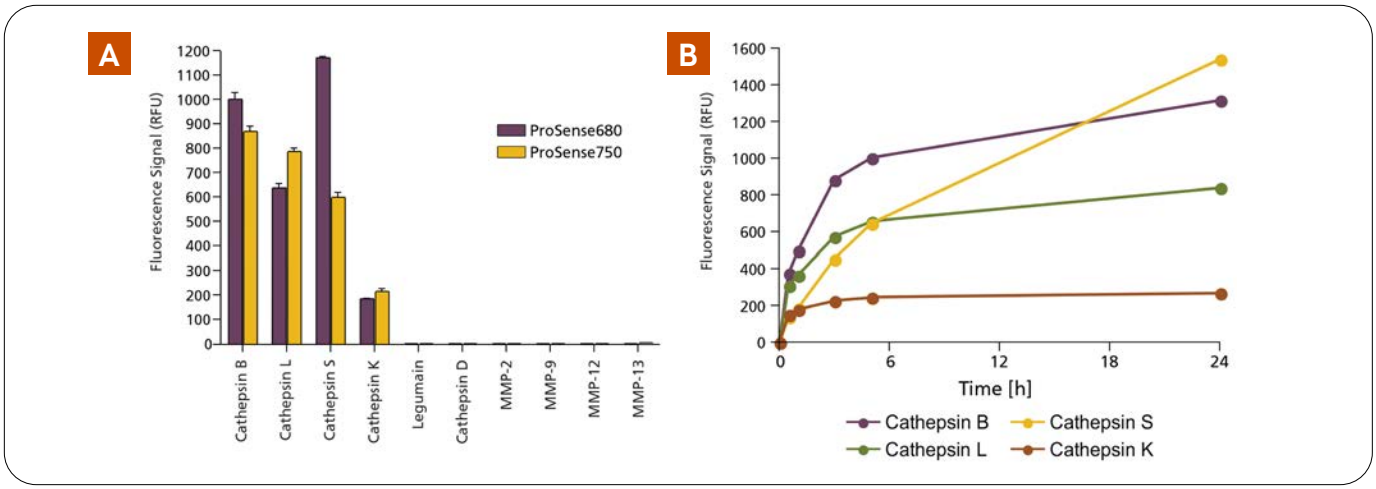


Figure 2: *In vitro* protease activation of IVISense Pan Cathepsin 680 and IVISense Pan Cathepsin 750 agents. The pan cathepsin fluorescent agents were incubated for up to 24 h in the presence of a variety of proteases known to be present in many tumors, including lysosomal proteases (cysteine proteases and legumain), an aspartyl protease (cathepsin D), and four secreted MMPs. A) Proteases were used to activate IVISense Pan Cathepsin 680 and IVISense Pan Cathepsin 750 agents in microwell enzyme assays to assess increases in fluorescence associated with cleavage of IVIS Pan Cathepsin agent at 24 h. B) The kinetics of cathepsin activation of IVISense Pan Cathepsin 680 agent is shown by assessing fluorescence at times 0, 0.5, 1, 3, 5, and 24 h following addition of recombinant protease.

IVISense Pan Cathepsin agent as a biomarker for cellular toxicity assays

To study cathepsin activity in conjunction with cell toxicity, HT-29 human colorectal adenocarcinoma cells (ATCC®, HTB-38™) were seeded at a density of 4,000 cells per well into a 96-well CellCarrier™ microtiter plate (Revvity®, 6005550) and left to adhere and grow for 72 h in serum supplemented medium. Cells were treated with various concentrations of 5-FU in serum supplemented medium and stained with 0.3 µM IVISense Pan Cathepsin 680 agent, 2 µM Hoechst, 1 µM BOBO™-3 and 5 µM YO-PRO®-1 to discriminate between live, apoptotic and dead cells. Before imaging, the staining/ treatment cocktail was removed from the cells and fresh 5-FU solution was added. 5-FU (also known as Adrucil, Carac, Efudix, Efudex and Fluoroplex) is a pyrimidine analog which is an established drug used in colorectal cancer treatment. Metabolites of this drug irreversibly inhibit thymidylate synthase, impair DNA repair and replication, incorporate into DNA to cause strand breakage and disrupt normal RNA function (Sat et al., 2003; Longley et al., 2003). The combined effects of 5-FU cause cells to undergo apoptosis and eventually caused cell death. Images were acquired using the Operetta® High Content Imaging System in non-confocal mode with the 40X LWD objective, under environmentally controlled conditions (37 °C and 5% CO_2) (Figure 3A). Stacks consisting of five planes with a distance of 3 µM were acquired at 72 and 96 h after treatment. Between measurements the plate was moved back into the incubator.

In order to detect live, dead and apoptotic cells across different focus heights, stack images were converted to maximum projection images using the corresponding stack processing method of the Harmony® High Content Imaging and Analysis Software. Three cell populations (live, apoptotic, dead) were detected individually on the corresponding channels. Live cells were detected on the Hoechst channel using the *Find Nuclei* Building Block of the Harmony software, while apoptotic and dead cells were not sufficiently stained with Hoechst. Once the cells become apoptotic, YO-PRO®-1 partially replaces Hoechst, resulting in green fluorescent nuclei, while dead cells are exclusively stained with the dead cell marker BOBO™-3. Therefore, it was required to detect apoptotic and dead cells on the YO-PRO®-1 and BOBO™-3 channels respectively. As final readouts, the numbers of live, apoptotic and dead cells were calculated.

Treatment with the anti-cancer drug 5-FU led to a strong increase in the number of apoptotic and dead cells. The quantification of YO-PRO®-1 positive cells showed a dose dependent induction of apoptosis, resulting in an EC_{50} of 88 µM (Figure 3B) under the culture conditions examined for this particular readout.

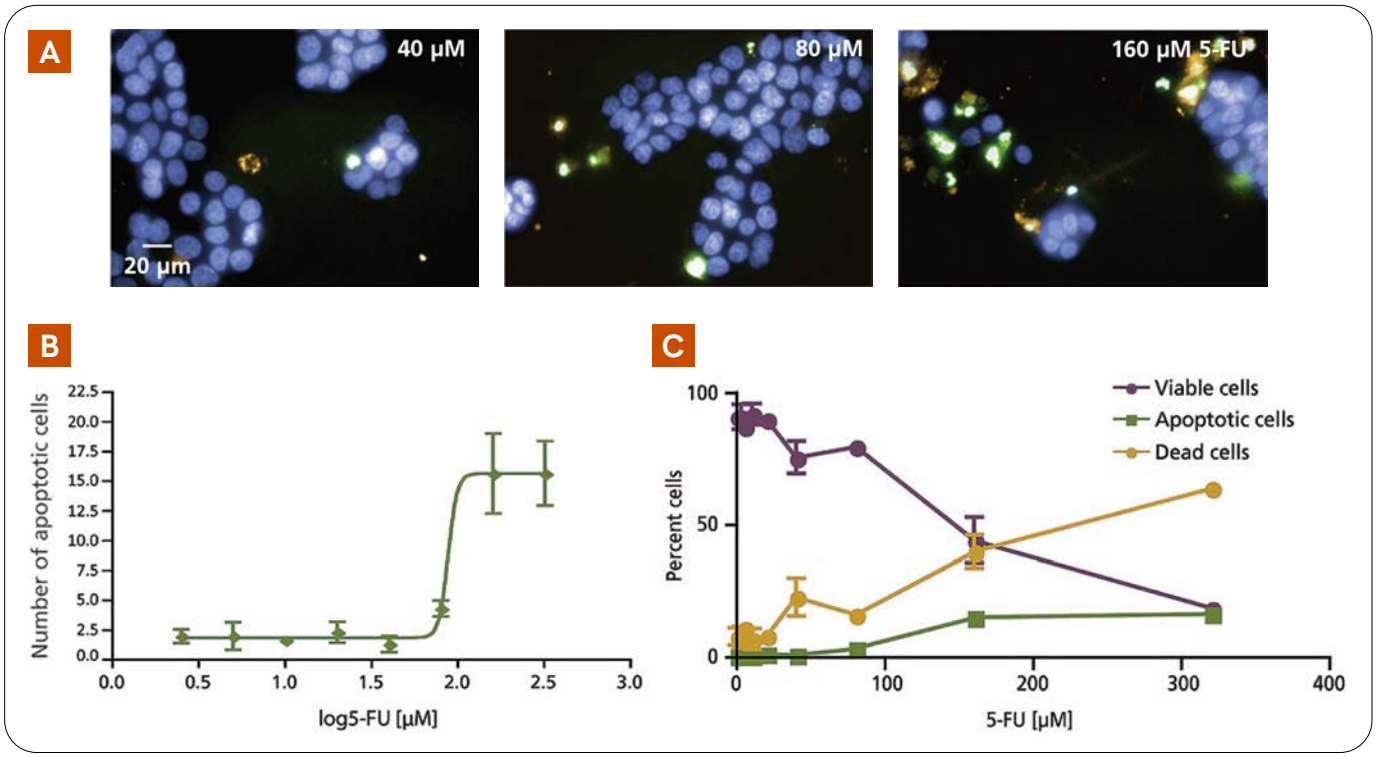


Figure 3: 5-FU induces apoptosis and cell death in HT-29 cells. Cells were incubated for 96 h in the presence of 5-FU. A) Representative images of HT-29 cells treated with 40 µM, 80 µM and 160 µM of 5-FU (around EC_{50}). Images show a strong increase in the number of apoptotic (YO-PRO®-1 positive, green nuclei) and dead (BOBO™-3 positive, yellow nuclei) cells with higher 5-FU concentrations. B) Incubation with 5-FU leads to a dose dependent increase in the number of apoptotic (YO-PRO®-1 positive) cells (EC_{50} = 88 µM). C) Cell classification into viable, apoptotic and dead cells shows a dose dependent decrease in the number of viable cells, while the number of apoptotic and dead cells increases (N = 3 wells).

In parallel to the Hoechst, YO-PRO®-1 and BOBO™-3 staining, HT-29 cells were labeled with IVISense Pan Cathepsin 680 agent to quantify cathepsin activity in the different cell populations. Incubation of HT-29 cells with IVISense Pan Cathepsin 680 agent leads to endocytic uptake followed by protease mediated activation of IVISense Pan Cathepsin agent in lysosomes, where cathepsin proteases reside inside the cell (Figure 4B).

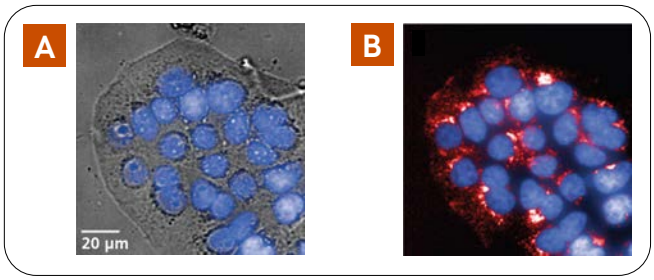


Figure 4: IVISense Pan Cathepsin 680 agent activation in viable HT-29 human colorectal adenocarcinoma cells. A) Overlay of brightfield and Hoechst (blue) images showing cells growing in a characteristic cell cluster. B) IVISense Pan Cathepsin 680 agent staining (red) after 72 h of incubation, showing cathepsin activity in the lysosomes of the cells.

Multiplexing of Hoechst, YO-PRO®-1, BOBO™-3 and IVISense Pan Cathepsin 680 agent cell labeling results in characteristic staining phenotypes. The IVISense Pan Cathepsin 680 agent is activated in viable cells and in a subpopulation of apoptotic cells, presumably early apoptotic cells that still show enzymatic activity. Little or no IVISense Pan Cathepsin 680 agent activation was observed in BOBO™-3 positive dead cells. To quantify IVISense Pan Cathepsin 680 agent signal in the different cell populations (live, apoptotic, dead), fluorescence was quantified in a ring region of 10 pixels surrounding the nuclei of all populations, created by the *Select Cell Region* Building Block. By introducing an intensity threshold in the Select Population Building Block of the Harmony software, IVISense Pan Cathepsin agent intensity was further used to distinguish between early and late apoptotic cells (Figure 5C). The absence of IVISense Pan Cathepsin agent activation in late apoptotic and dead cells clearly shows that a viable, functioning cell is required for optimal cathepsin activity.

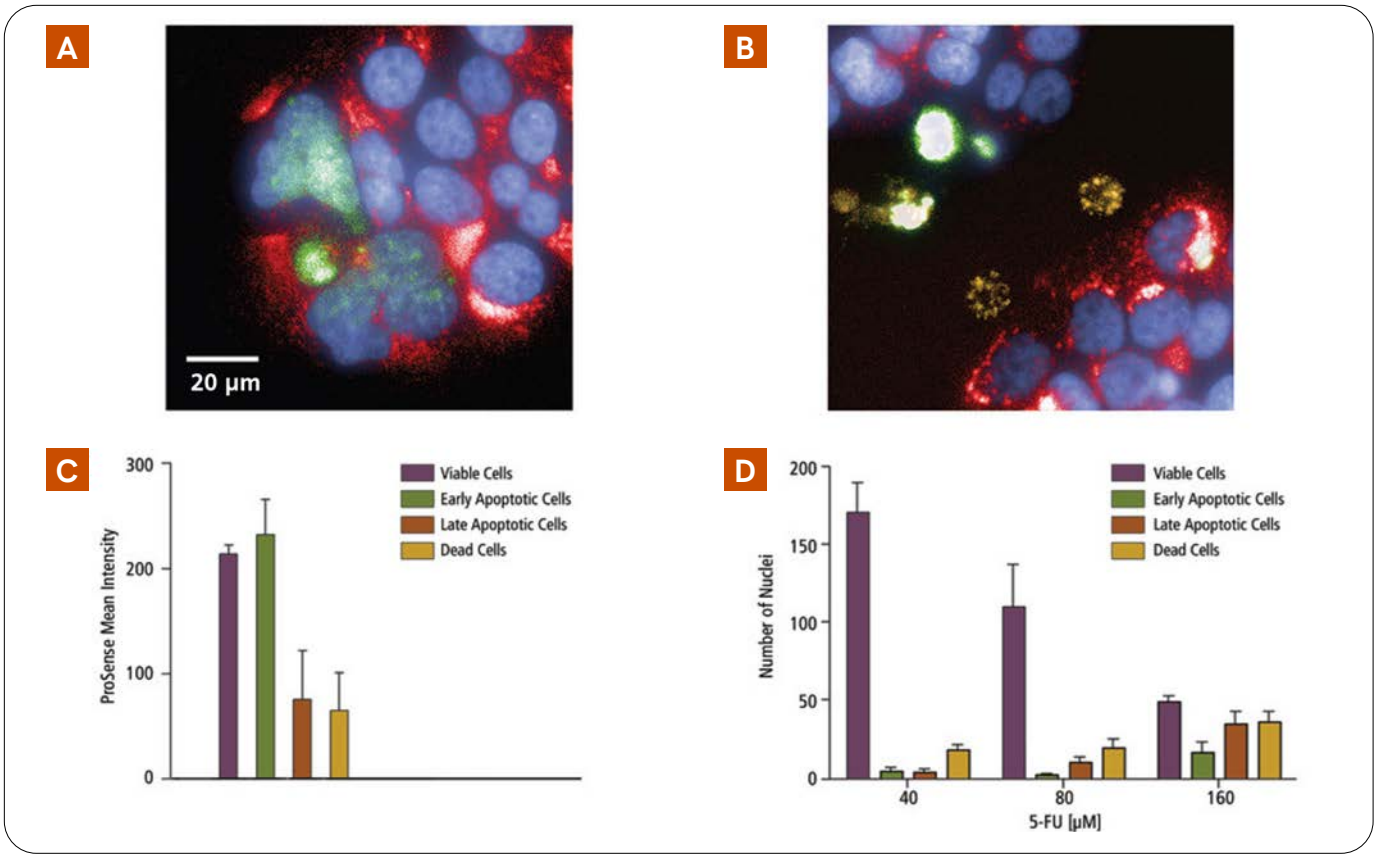


Figure 5: IVISense Pan Cathepsin 680 agent is activated in live and early apoptotic cells but not in late apoptotic and dead cells. Live HT-29 cells were incubated with a treatment/staining cocktail containing 80 µM 5-FU, Hoechst (blue), YO-PRO®-1 (green), BOBO™-3 (yellow) and IVISense Pan Cathepsin 680 agent (red) for 72 h. A) Viable cells show a Hoechst signal (blue) in the nucleus and IVISense Pan Cathepsin 680 agent activation (red) in the cytoplasm. Early apoptotic cells are still part of cell clusters and are characterized by YO-PRO®-1 positive nuclei (green) and IVISense Pan Cathepsin 680 agent activation (red). B) Late apoptotic cells show YO-PRO®-1 positive nuclei (green) but lack a IVISense Pan Cathepsin 680 agent signal and dead cells are exclusively labeled with BOBO™-3 (yellow). C) IVISense Pan Cathepsin 680 agent intensity differs between cell populations and distinguishes between early apoptotic and late apoptotic cells. D) Classification of cells into viable, early apoptotic, late apoptotic and dead cells following treatment with 40 µM, 80 µM and 160 µM 5-FU. Increasing concentrations of 5-FU shift the cell populations towards more apoptotic and dead cells (N = 3 wells).

INVSense Pan Cathepsin agent as a non-invasive imaging biomarker for tumor progression and therapy

To study tumor cathepsin activity in an *in vivo* mouse model of human cancer, HT-29 human colorectal adenocarcinoma cells were implanted (3.5 x10^6 cells per site) into both upper mammary fat pad regions of 5-6 week old nu/nu mice (n = 5 mice per group). Mice were maintained in a specific pathogen-free animal facility and provided with water and low-fluorescence mouse chow. Handling of mice and experimental procedures were in accordance with veterinarian requirements for animal care and use. Mice received either no treatment or daily intraperitoneal (i.p.) injection with 5-FU from day 1 to day 2 (35 mg/kg/day) and day 3 to day 11 (20 mg/kg/day). Progression in tumor size over 2 weeks post-implantation, with and without treatment, was assessed by standard vernier caliper measurement of the width of each tumor along 2 axes, from which tumor volume was calculated. Figure 6 shows that 5-FU treatment of HT-29 tumor-bearing mice significantly impacted the progression of the tumors (47% decrease by day 14) as measured by calipers (Figure 6C), and this trend was seen as early as day 10.

At 14 days post-HT-29 implantation, when the control, untreated tumors reached an average volume of ~180 mm^3, all mice were injected with 2 nmol of IVISense Pan Cathepsin 680 agent and imaged 24 h later. This time point for imaging allowed for full tumor lysosomal uptake and activation of the fluorescent imaging agent. IVISense Pan Cathepsin 680 agent is able to detect cathepsin activity associated with tumor associated macrophages (TAM), a cell population that can increase dramatically at some early time points during a treatment regimen.

However, this particular study was deliberately designed not to address TAM-associated cathepsin activity and capture a time posttreatment at which the innate immune response was expected to decline. Indeed, significant treatment-associated decreases in IVISense Pan Cathepsin 680 agent fluorescence occurred as shown using fluorescence molecular tomography. Epifluorescence imaging (Figure 6A), which detects surface fluorescence signals, and tomographic imaging (Figure 6B), which detects 3-dimensional fluorescent signals distributed throughout the animal, were both highly effective in detecting decreases in signal associated with drug treatment. Quantitative analysis of tomographic imaging datasets was performed by minimal thresholding (30% of the mean nM concentration of the control tumors) to remove background fluorescence in the tumor regions, which could compromise assessment of tumor-associated signal. Fluorescence, quantified as either the volume of tumor fluorescence (Figure 6D) or the total tumor fluorescence in pmol (Figure 6E), showed a decrease in parallel with physical measurements of tumor size using calipers (Figure 6C).

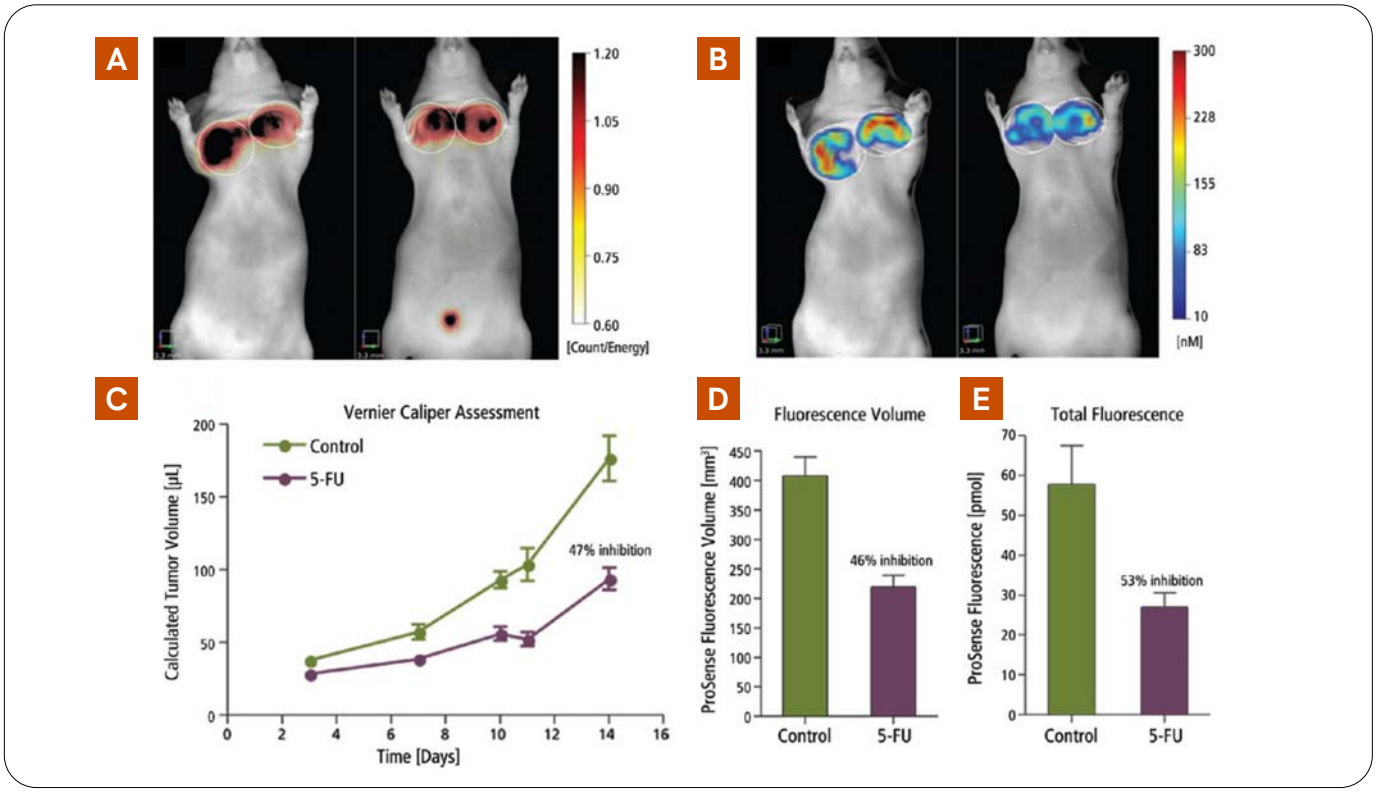


Figure 6: *In vivo* imaging of 5-FU treatment efficacy in HT-29 tumor xenografts using IVISense Pan Cathepsin 680 agent. Mice bearing HT-29 tumor xenografts, control (left) and 5-FU- treated (right), were injected intravenously with IVISense Pan Cathepsin 680 agent (2 nmol per mouse) and imaged 24 h later on the FMT 2500 system. Images show representative mice, imaged by A) epifluorescence and B) fluorescence tomography. C) Treatment with 5-FU decreased tumor size by approximately 47% as assessed by physical tumor measurement (vernier caliper assessment). D) Imaging using the IVISense Pan Cathepsin 680 agent revealed similar decreases in tumor fluorescence volume and in **E)** total fluorescence per tumor (N = 5 mice per treatment group).

Conclusion

In this study we assessed and validated the use of our cathepsin-activatable IVISense Pan Cathepsin 680 fluorescent imaging agent in *in vitro* and *in vivo* assays, and further defined its usage in measuring drug treatment efficacy. IVISense Pan Cathepsin agent was used as a fluorogenic substrate in a panel of protease assays, revealing optimal activation in 3-5 h and selectivity for cysteine cathepsin family members. HT-29 cells derived from a human colon adenocarcinoma showed uptake and activation of the IVISense Pan Cathepsin agent into lysosomes as assessed using the Operetta system, a finding consistent with the known biology of cysteine cathepsins.

Correlation of IVISense Pan Cathepsin agent intensity with independent fluorescent markers of live, apoptotic and dead cells revealed that cathepsin activity declines in 5-FU-induced late apoptosis and is absent in cells that die in response to treatment. This places cathepsin biomarkers in the category of cell metabolism markers, and suggests their value as an accurate discriminator between early and late apoptosis. The activatable nature of the IVISense Pan Cathepsin agent allows visualization of enzyme activity rather than just the enzyme localization. Therefore, it provides a much higher degree of biological information compared to targeted agents such as antibodies that are often used in imaging assays. *In vivo* imaging using the FMT 2500 system is in agreement with *in vitro* findings made using the Operetta system and shows the ability to measure 5-FU efficacy in treating human colorectal tumor xenografts. Non-invasive fluorescence imaging of nude mice bearing HT-29 tumor xenografts showed decreased fluorescence (pmol and volume) upon 5-FU treatment that correlated well with tumor size as assessed by vernier caliper measurements. Importantly, since the IVISense Pan Cathepsin agent is only activated in viable and early apoptotic cells, it is a useful tool to label and study living cancer cells; IVISense Pan Cathepsin agent quantification will not include necrotic or cystic tumor regions which have been found to confuse interpretation when using tumor size measurements in efficacy readouts.

With this study we provide an illustration of the potential translational use of a cathepsin activatable agent – from well to cell to animal – to close the gaps between biochemical, *in vitro* and *in vivo* experiments in oncology research. By using the same agent in all assays, biological interactions are comparable, and consequently translational experiments have the potential to increase the reliability for the drug discovery process.

References

Bremer, C., Tung, C.H., Bogdanov, A. Jr., Weissleder, R. (2002): Imaging of Differential Protease Expression in Breast Cancers for Detection of Aggressive Tumor Phenotypes. *Radiology*, 222, 814-818.

Conus, S., Simon, H.U. (2010): Cathepsins and their involvement in immune responses. *Swiss Med. Wkly.,* 140, w130-142.

Katunuma, N. (2010): Posttranslational Processing and Modification of Cathepsins and Cystatins. Journal of Signal *Transduction,* 2010, 375-345.

Longley, D.B., Harkin, D.P., Johnston, P.G. (2003): 5-fluorouracil: mechanisms of action and clinical strategies. *Nat. Rev. Cancer,* 3, 330-338.

Mohamed, M.M., Sloane, B.F. (2006): Cysteine cathepsins: multifunctional enzymes in cancer. *Nat. Rev. Cancer,* 6, 764-775.

Sat, B., Reches, M., Engelberg-Kulka, H. (2003): The Escherichia coli mazEF suicide module mediates thymineless death. *J. Bacteriol.*, 185, 1803-1807.

Turk, V., Turk, B., Turk, D. (2001): Lysosomal cysteine proteases: facts and opportunities. *EMBO J.,* 20, 4629-4633.

Vasiljeva, O., Reinheckel, T., Peters, C., Turk, D., Turk, V., Turk, B. (2007): Emerging roles of cysteine cathepsins in disease and their potential as drug targets. *Curr. Pharm. Des.,* 13, 387-403.

Zavasnik-Bergant, T., Turk, B. (2006): Cysteine cathepsins in the immune response. *Tissue Antigens*, 67, 349-355.

Authors

Marcel Waschow
Karin Boettcher
Jeffrey D. Peterson

Revvity, Inc.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com