

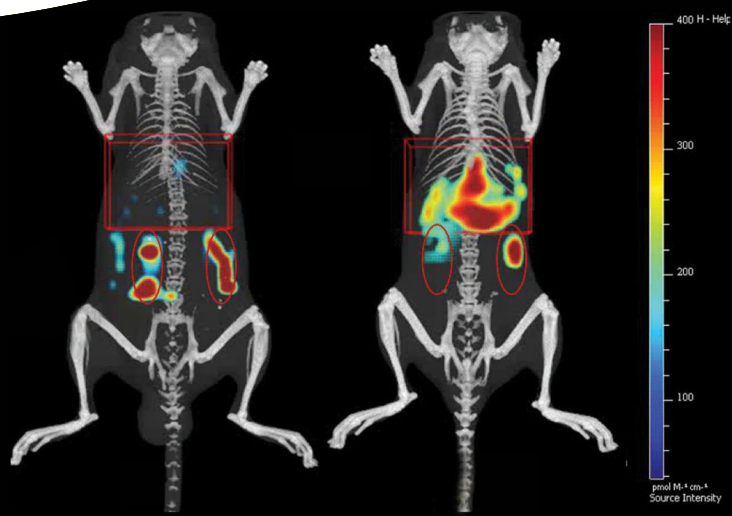
Imaging hepatocellular liver injury using NIR-labeled Annexin V.

Abstract

Drug induced liver injury (DILI) is a major reason for late stage termination of drug discovery research projects, highlighting the importance of early integration of liver safety assessment in the drug development process. A technical approach for *in vivo* toxicology determination was developed using Acetaminophen (APAP), a commonly used over-the-counter analgesic and antipyretic drug, to induce acute hepatocellular liver injury. Revvity imaging technology and near infrared (NIR) labeled Annexin V (IVISense™ Annexin-V 750) were used to detect and quantify and necrosis associated with this type of liver toxicity. Both fluorescent tomographic imaging (FMT® 4000 and IVIS® SpectrumCT), and higher throughput epifluorescence imaging (IVIS SpectrumCT), provided excellent detection of IVISense Annexin-V fluorescence in the liver. Histology and plasma alanine transaminase (ALT) confirmed the kinetics of tissue necrosis, and more extensive liver damage was seen but with an apparent decrease in tissue PS and plasma ALT by 48 hours, suggesting a decline in the induction of tissue destruction. Compared to conventional plasma/serum assays, *in vivo* imaging can offer fast, quantitative imaging results directly assessing the tissue of interest.

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Materials

Fluorescent agent

IVISense Annexin-V 750 (AV750) fluorescent agent is specific for phosphatidyl serine exposed on the surface of cells undergoing either apoptotic or necrotic cell death. This agent was designed to non-invasively detect and image cellular death occurring due to tumor chemotherapy, adverse drug effects on normal tissue, or spontaneous cell death associated with disease progression in mice. The imaging dose for this agent was as recommended in the product insert (2 nmol/25 g mouse).

Mice for APAP - induced liver injury model

Seven to eight week-old male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a pathogen-free animal facility with water and low-fluorescence mouse chow (Harlan Tekland, Madison, WI). Handling of mice and experimental procedures were in accordance with Revvity IACUC guidelines and approved veterinarian requirements for animal care and use. The animals are fasted overnight (approximately 18 h) prior to drug administration.

Table 1. Basic properties of IVISense Annexin-V 750 fluorescent tumor imaging agent.

Agent Summary. Characteristics of the agent (MW/size, excitation/emission [Ex/Em], and blood/tissue pharmacokinetics) were determined in multiple independent studies.

IVISense Annexin-V 750	
Agent type	Phosphatidyl Serine Targeted Agent
Molecular weight	34,000 g mol ⁻¹
Ex/Em	755/772 nm
Blood half-life	Distribution t _{1/2} ~30 min
Tissue half-life	~14 h

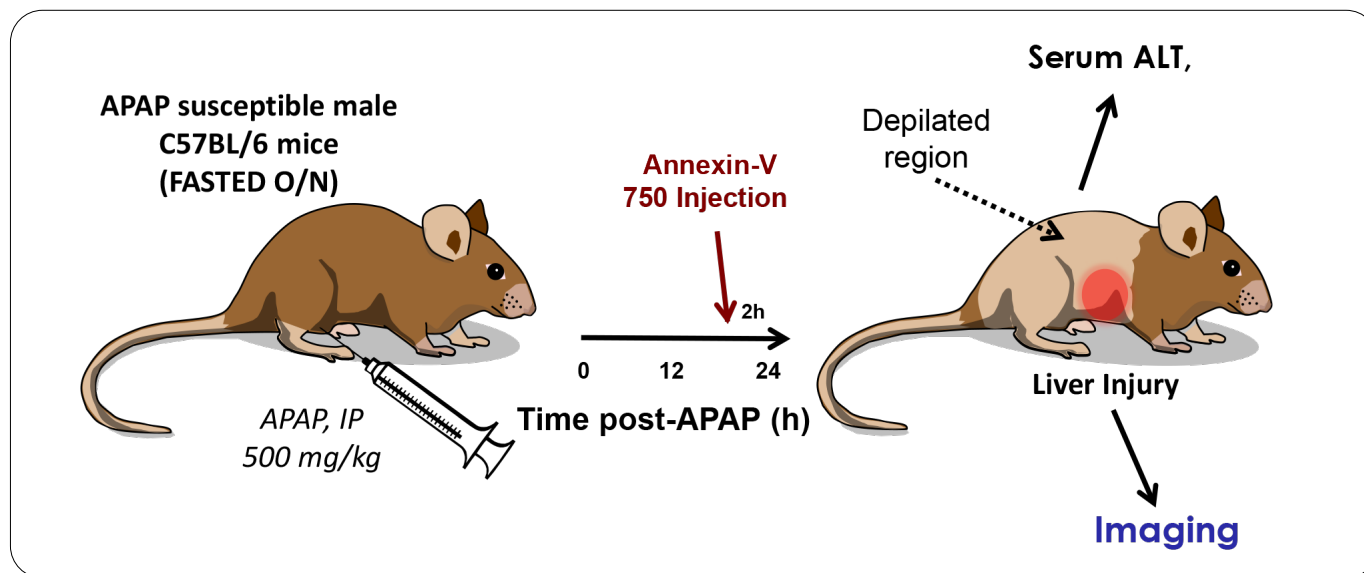


Figure 1. Optimal Imaging Protocol for Hepatocellular Liver Injury

Hepatocellular injury imaging protocol

Preparing mice for imaging

- Use 7-8 week old C57BL/6 male mice for APAP-induced liver injury, as this particular drug response is very mouse-strain and gender dependent. Other drugs that induce similar liver injury may differ in mouse strain/gender dependence or be independent of these variables.
- Two weeks before the imaging study, switch to low fluorescence mouse chow. Regular mouse chow contains chlorophyll that auto-fluoresces around 700 nm and can interfere with signal collected from this agent.
- Animal hair is highly effective at blocking, absorbing, and scattering light during optical imaging. Even light within the NIR spectrum, which typically shows minimal scattering and absorbance in tissue, is significantly absorbed and scattered by hair.
- Always remove the fur on and around the areas of the animal that are to be imaged (Nair depilatory lotion; Church & Dwight Co, Princeton NJ), whether performing 2D or 3D optical imaging, within 1-24 h prior to imaging. Take care to minimize the time of Nair treatment and rinse mice well with warm water. IP injection of ketamine/xylazine (100 mg/kg and 20 mg/kg, respectively) may be needed to keep mice anesthetized for the procedure.
- Mice should be fasted overnight (~18h) for most consistent induction of liver injury. Water is provided.

Preparing IVISense Annexin-V 750

- Each vial contains 10 mouse doses of IVISense Annexin-V 750 in solution (Total volume 1 mL). This material provides sufficient reagent for imaging approximately 10 mice (weighing ~25 grams each) when using the recommended dose of 100 µL of IVISense Annexin-V 750 per mouse.
- IVISense Annexin-V 750 is stable for up to six months when stored at 2-8°C and protected from light.

Introducing hepatocellular liver injury with APAP

- APAP must be freshly prepared from stock powder as a 300-500 mg/kg solution (20 mg/ml in PBS) for effective induction of liver injury. Warming the mixture to 55°C for 10 minutes may be required for generation of a homogeneous solution.
- Fasted mice are injected IP with a single, bolus dose of either 375-625 µl APAP or PBS (control group) in a 20 g mouse. Optimal peak induction of liver cellular death is around 24 h for this particular treatment.

Animal imaging considerations

At the desired time (recommended 22 h post-APAP), inject 100 µl of AV750 intravenously into APAP and control mouse groups. Other drugs that induce hepatocellular injury may require establishing the optimal timecourse post-drug or even several days of dosing prior to AV750 injection.

- The optimal imaging time for AV750 is two hours post injection. The optimal re-injection time is every 1-2 days, which allows for the clearance of the agent from the liver. It is important to note that kidney signal can be retained somewhat longer but with minimal impact on liver imaging.

IVIS Spectrum optical imaging

- If using the epifluorescence (2D) feature of an IVIS imaging system, place the anesthetized animal in the supine position (i.e. belly-up) to facilitate optimal liver signal detection. The systems provide gas anesthesia to the imaging chamber.
- For the IVIS Spectrum and IVIS SpectrumCT, the 745 ex/800 em filter set was used. For the IVIS Lumina we used either 745 ex/ICG em (standard Lumina filter set) or 745 ex/800 em.
- For rapid animal screening, IVIS systems permit imaging of five mice at the same time using Field of View D (FOV D). It is essential to use guards between animals to prevent signal contamination from neighboring animals.
- For the IVIS Spectrum and IVIS SpectrumCT, optical tomography (FLIT) can also be used, with imaging of one mouse at a time. Transillumination scan fields should be established with 15-20 scan points covering the liver and some area above and below the liver. This will take 12-15 minutes for acquisition.
- Reconstruction of 3D datasets should be performed using appropriate thresholding and masking out of regions far outside the scan field prior to reconstruction. Images should be represented with 0.62 mm voxel size and with optimal color scales for appropriate 3D fluorescence representation.
- Liver quantification in APAP and control mice is performed by placement of 2D or 3D regions of interest (ROI) to capture the fluorescent signal within the liver region.
- For further information on 2D (epifluorescence) or 3D (FLIT) IVIS imaging, please refer to tech notes under the help tab of the Living Image® (LI) software.

FMT 4000 Imaging

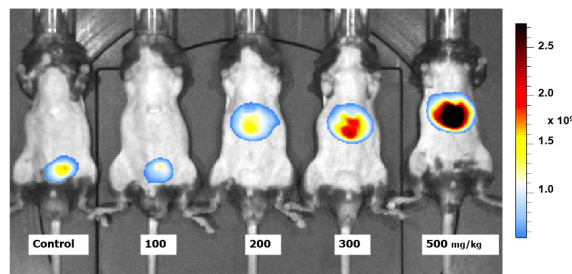
- The FMT 4000 imaging system is a dedicated fluorescence tomography system, but epifluorescence images are also acquired at the same time. As with IVIS imaging, it is important to position mice in the supine position, in this case within an animal imaging cassette.
- Use the height adjustment knobs on the cassette to make sure that the anesthetized animal is gently but securely compressed between the top and bottom plates.
- Slide the cassette into the docking station with the animal in the prone position. The system provides gas anesthesia to the cassette within the imaging chamber. Set up the scan field with 30 to 50 scan points with at least 1-2 rows of scan points surrounding (on all sides) the liver region. Acquisitions typically take 5-7 minutes.
- Select AV750 as the imaging agent, which will select the optimal laser and filter combination for 750 nm imaging.
- 3D reconstructions are performed automatically by TrueQuant software. Images should be represented with optimal thresholding and optimal color scales.
- Liver quantification in APAP and control mice is performed by placement of 3D ROIs to capture the fluorescent signal within the liver region.

Introduction and results

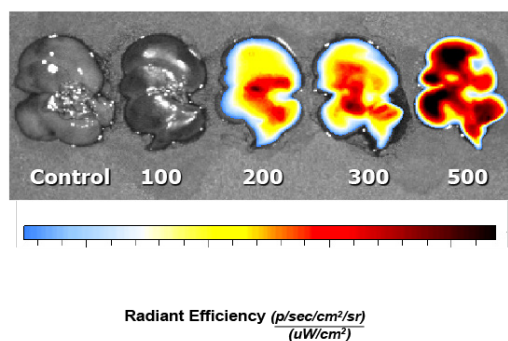
Drug Induced Liver Injury (DILI) has been the most frequent single cause of safety-related drug withdrawals from the marketplace for the past 50 years and continues to be an important consideration in drug discovery research today. Liver safety issues further limit use of many approved drugs or prevent consideration for approval. The majority of drugs showing evidence of DILI show hepatocellular toxicity, a form of liver injury with cellular death and little or no evidence of hepatobiliary obstruction, cholestasis, or steatosis. The most overtly toxic of such drugs will cause DILI in anyone receiving a high enough dose, however many drugs (at recommended doses) show adverse liver finding in <1 per 10,000 individuals.

Hepatocellular injury can be caused by drugs that can cause severe DILI (like carbon tetrachloride) as well as by drugs that rarely cause severe DILI, such as aspirin, tacrine, statins, and heparin. Yet both types of drugs can cause significant elevations in serum ALT or AST. Revvity's optical imaging technologies and *in vivo* imaging agents offer a unique approach to detecting *in situ* liver damage in living mice rather than relying on serum biomarkers or terminal histology.

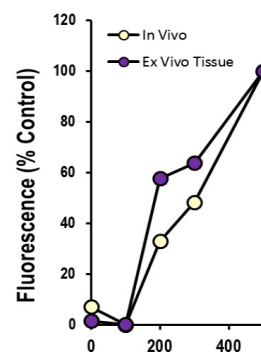
A In Vivo imaging



B Liver imaging



C Imaging quantification



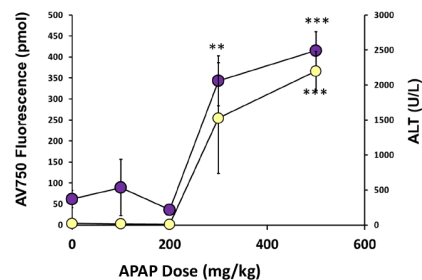
Whole mouse epifluorescence imaging was used to detect accumulation of AV750 following APAP treatment.

- Epifluorescence images of mice receiving different doses of APAP 24 h prior to imaging shows an increase in signal with APAP dose.
- Epifluorescence imaging of excised livers from APAP treated mice.
- Quantification of liver signal from non-invasive imaging and ex vivo imaging was determined by ROI placement to capture the entire liver, and results were represented as the percent of the 500 mg/kg group.

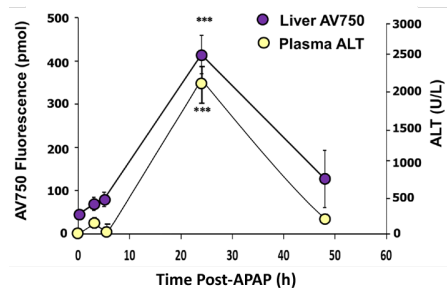
Figure 2. IVIS Imaging

Pairing either the IVIS SpectrumCT or the FMT 4000 with the imaging agent IVISense Annexin-V 750 (AV750) allows the imaging and detection of cellular death within the liver. Acetaminophen (APAP), a commonly used over-the-counter analgesic and antipyretic drug, is known to cause centrilobular hepatic necrosis when used at high doses. When male C57BL/6 mice were fasted overnight and injected with a single dose (200-500 mg/kg) of APAP, the resulting liver necrosis peaked at 24 hours as detected by imaging with AV750 in living animals (Fig 1). Dose ranging of APAP (100, 200, 300, and 500 mg/kg) showed maximal tissue destruction at 300 and 500 mg/kg by AV750 (Fig 2) and confirmed by histology and serum ALT (Fig 3). Imaging results at the 24 h were statistically significant as compared to those of the PBS control group using only 3-4 mice per group. Histology and serum alanine transaminase (ALT) confirmed the kinetics of tissue necrosis, and at 48 hours more extensive liver damage was seen but with an apparent decrease in tissue PS and serum ALT by 48 hours, suggesting a decline in the induction of tissue destruction (Fig 4). Both fluorescent tomographic imaging (FMT 4000 and IVIS SpectrumCT), provided excellent detection and quantification of AV750 fluorescence in the liver (Fig 5). Compared to conventional plasma/serum assays, *in vivo* imaging can offer fast, quantitative imaging results directly assessing the tissue of interest. Our results to date demonstrate the potential of optical imaging for assessing compound liver toxicity in early drug discovery programs.

A Dose response 24 h



B APAP Time course (APAP 500 mg/kg)

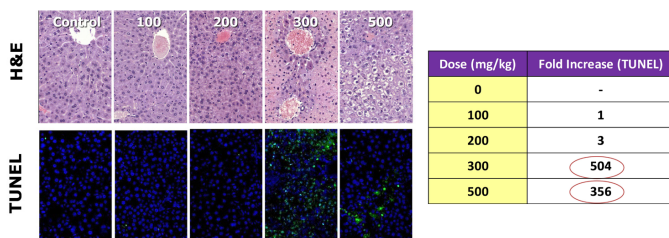


Non-invasive *in vivo* AV750 fluorescence imaging results were compared to serum alanine transaminase (ALT) levels.

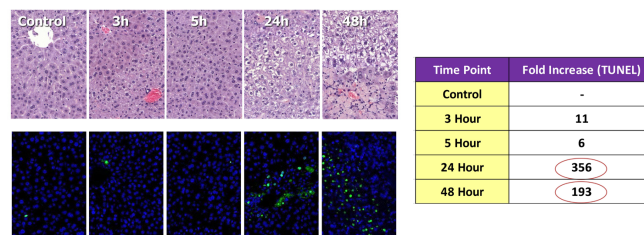
- A. Fluorescence imaging and serum ALT dose response to APAP treatment at 24 h.
- B. Fluorescence imaging and serum ALT time course of response to 500 mg/kg of APAP treatment. Asterisks indicate statistically significant differences between untreated controls and APAP-treated groups (**p < 0.001; ***p < 0.005).

Figure 3. Comparison to Serum ALT

A Dose Response (24 h)



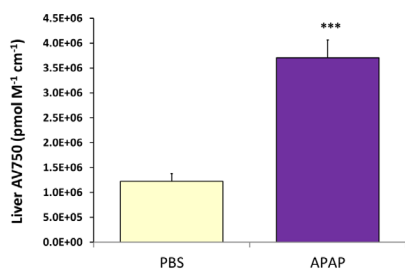
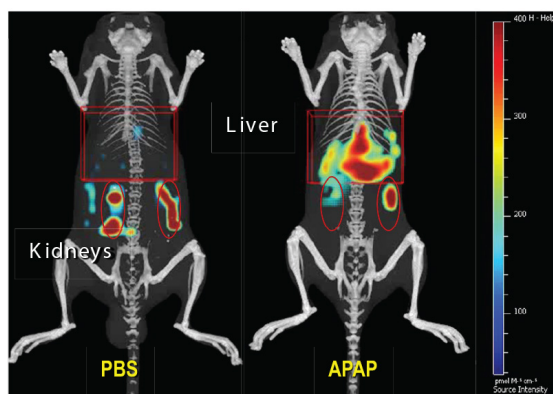
B APAP Time course (APAP 500 mg/kg)



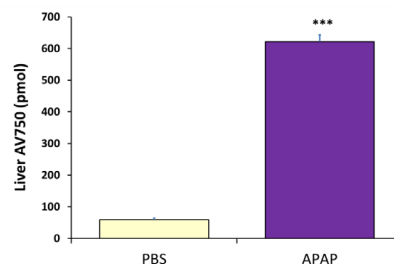
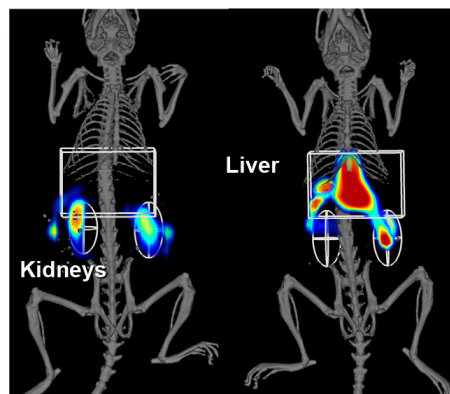
Images are shown from representative tissue regions/sections for APAP dose response (A) and time course (B) studies. Formalin-fixed, paraffin-embedded tissue sections from APAP treated mice were assessed by H&E (upper panels) and fluorescent TUNEL staining (lower panels). Representative images were acquired on the Vectra® Imaging system and spectral unmixing was used to specifically eliminate background autofluorescence by spectral signature. Lower panels show the corresponding fold increases of apoptosis in liver sections using InForm® advanced image analysis software.

Figure 4. Histology Corroboration

A 3D IVIS FLIT/CT imaging



B 3D FMT 4000 Fluorescence imaging with Quantum μCT co-registration



APAP-treated (500 mg/kg) and control mice were also assessed using 3D tomographic imaging on the IVIS Spectrum (A) and FMT 4000 (B) imaging systems. The IVIS SpectrumCT data was acquired with 15 minute scans of the liver region, including the built in CT functionality. In a separate study, FMT 4000 acquisitions of the liver region were acquired with 5 minute scans and separate <1 min uCT scans on the Quantum (Revvity). DICOM files were exported and combined as a co-registered image of fluorescence and microCT data. Fluorescent signal in the kidneys could also be detected due to the known clearance of Annexin V through this route. Bar charts below represent the quantitation of imaging results, and asterisks indicate statistically significant differences between control and treated groups (***p < 0.005).

Figure 5. Tomographic Fluorescence Imaging

Conclusions

The present studies provides evidence that APAP induced liver injury can be quantified non invasively *in vivo* with IVISense Annexin-V 750 fluorescent probe used in conjunction with Revvity's imaging technologies. Rapid 2D epifluorescence imaging of 5 mice at a time provides a robust and quantitative method for assessing hepatocellular toxicity in the liver in living mice, and the results correlated well with direct imaging of excised liver fluorescence. The time course and dose-response of APAP - induced liver

injury were quantified by imaging, and the results correlated well with serum ALT levels, direct imaging of excised livers, qualitative assessment of liver damage by H&E staining of tissues, and the increase in TUNEL staining. Tomographic (3D) imaging provided additional depth detection and detail of signal within the liver, as well as detection of the expected kidney clearance of Annexin V.

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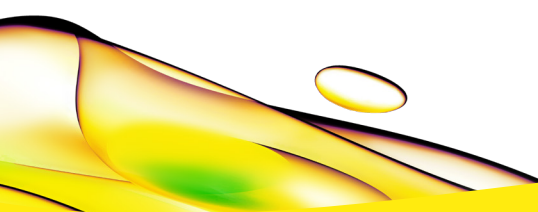
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