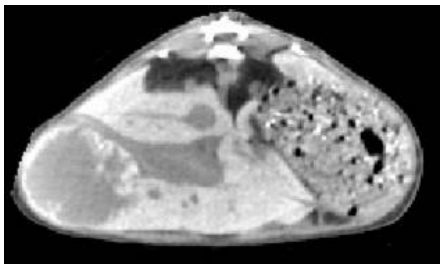

Fenestra® LC User Guide with MicroCAT II Imaging Hardware

Hepatic Imaging of in C57Bl/6 *Mice: Visualization of Hepatic Focal Lesions and Anatomy Using a MicroCAT II (ImTek, Inc.) Scanner*

- ***Proven Technology***
- ***Powerful Image Enhancement***
- ***Wide Applicability in Research and Drug Development***

In this guide:

Technology Overview

MicroCT Imaging

A miniaturized version of traditional clinical computed tomography (CT) imaging systems known as microCT has been developed in recent years that provides exceedingly high resolution images. Though initially used almost exclusively in *ex vivo* applications, recent advances in imaging hardware and computing power have extended the utility of the technique to include living animals at isotropic spatial resolutions approaching 5-10 μm. Despite being able to provide this incredible spatial resolution, however, two key problems currently hinder the widespread adoption of the method in living systems: 1) the relative lack of soft tissue contrast associated with taking such thin tomographic slices complicates interpretation of anatomical features; and 2) comparatively long acquisition times preclude the use of standard water soluble iodinated contrast media that might otherwise overcome the problem of inherently low soft tissue contrast.

The Fenestra Solution

MediLumine, Inc. has licensed a technology developed by scientists originally at the University of Michigan but currently at the University of Wisconsin that solves several key problems associated with contrast enhancement in microCT imaging. The technology is comprised of iodinated lipids that provide contrast enhancement and a novel oil-in-water lipid emulsion that selectively localizes the lipids to various locations within the body.

MediLumine's Fenestra® LC provides visualization of the hepatobiliary system by exploiting the endogenous lipid metabolism pathways present in the body. Chylomicron remnants (CMR) represent a class of naturally-occurring plasma lipoproteins that selectively shuttle lipids to hepatocytes in the liver. Fenestra LC mimics CMR particles and thereby localizes the contrast-producing lipids it contains into the liver's parenchyma following intravenous administration. Because the uptake and clearance profiles of the lipid molecules are determined by the metabolic status of extracellular and intracellular liver lipases, Fenestra LC provides the ability to assess both hepatobiliary anatomy as well as liver

function by CT imaging. In normal animals, hepatic contrast enhancement lasts for up to several hours after injection. Moreover, since the contrast-carrying metabolites of Fenestra LC are eliminated into the bile, image enhancement of the gastrointestinal tract is possible as well.

Fenestra® VC is a refined version of Fenestra® LC in which the surface of the lipid emulsion particles is modified so as to alter the recognition of the particle by the receptors on hepatocytes that are responsible for its uptake into the liver. With Fenestra VC, the delayed uptake by liver cells produces an agent with superior blood pool imaging properties that last for several hours after injection. Moreover, the agent remains truly intravascular so long as the endothelial integrity of the vessel is maintained. Like its liver-selective counterpart, Fenestra VC is eventually metabolized and eliminated through the hepatobiliary system.

Because of their comparatively long and stable *in vivo* residence times (up to several hours), the Fenestra® products have shown considerable promise for use in microCT imaging procedures. The numerous benefits provided by the Fenestra technology will play an instrumental role in facilitating the implementation of microCT imaging as an increasingly important and popular component of both basic research and commercial drug development.

Types of Studies

MediLumine's Fenestra® product line can be used in a wide range of CT imaging applications. To date, seven different animal species (mouse, rat, rabbit, dog, pig, monkey and woodchuck) have been studied successfully using the agent in numerous normal and disease model conditions. Fenestra LC has already been used to visualize normal and diseased hepatic parenchyma, biliary function and anatomy, focal liver lesions (tumors, ablations, etc.) and even hepatic tumor vasculature in living animals. Fenestra® LC could also be used to non-invasively assess tumor-induced angiogenesis and the effectiveness of anti-angiogenesis drugs on hepatic primary or metastatic lesions. Importantly, using Fenestra LC, these studies can be performed with the tumor of interest located in the actual microenvironment that it would see clinically – the liver – rather than in a distant and potentially unrealistic flank or peripheral location. Clearly, Fenestra LC provides substantial flexibility in the type and scope of studies that can be performed.

Key Benefits and Features of Fenestra® LC

A number of important features of Fenestra® LC make it the imaging agent of choice for your CT imaging studies. Among the primary benefits of the agent are:

- Prolonged contrast enhancement of the entire hepatobiliary system from a single administration
- Intracellular localization in hepatocytes correlated to metabolic status of the liver
- Simultaneous anatomical and functional assessments possible
- Repeat administration without safety concerns
- Pharmacokinetic profile compatible with microCT imaging temporal constraints
- Physicochemical properties compatible with parenteral administration

The combination of benefits provided by Fenestra LC allow for improvement of studies currently being conducted using CT imaging as well as design and conduct of studies that are technically challenging or even impossible using other imaging agents.

Storage and General Use Information for Fenestra® LC

Fenestra LC should be stored at room temperature (20-25 ºC) and should never be frozen. The visual appearance of Fenestra LC should be a white or slightly off-white milky fluid. Do not use the agent if the formulation appears to have separated into separate oil and water phases. Fenestra LC is provided in a multi-use vial and should be used before the expiration date on the vial label. Prior to use, the vial should be mixed by gentle inversion or shaking. If the agent is not used within 5-10 minutes of drawing the dose, the contents of the syringe should be remixed by gentle inversion immediately prior to injection.

***Example of Fenestra® LC Use*: Imaging of Hepatic CT-26 Tumors in Balb/c Mice as a Model of Metastatic Liver Cancer**

Animals

a. Strain: Balb/c mice bearing solitary hepatic CT-26 tumors
b. Characterization: 20-21 g males
c. Animal model: A solitary CT-26 tumor (adenocarcinoma of colonic origin) was created in the liver of each of three anesthetized male Balb/c mice by direct injection of $5x10^5$ cells in 50 µl PBS into the exposed medial lobe of the liver through a 30-ga needle. Stasis was achieved with light pressure at the site of needle insertion. The laparoscopic incision was closed with surgical staples, which must be removed prior to imaging to avoid image artifacts. Tumors reach a size of 2-3mm in diameter within 5 days of implantation.

Animal Prep

a. Fasting/GI preparation: MicroCT imaging is best performed in mice that have been maintained on a non-chow, soft (vegetable or liquid) diet for 24-48 hours prior to study to minimize imaging artifacts due to minerals that are found in rodent chow (see abdominal region in Image 1). While fasting is an alternative to the soft diet, clearance of digested chow from the GI tract may be incomplete.
b. Anesthesia: For this study, mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight) affording 45-60 min of anesthesia. Maintenance of anesthesia was achieved with quarter dose increments as needed during the duration of the study. Choice of anesthetic should be based upon individual protocols, however, consideration should be given to the desired level of anesthesia, duration of anesthesia required for completion of the study, how the anesthetic affects on respiration rate and cardiac function may influence image quality (isoflurane inhalation) and the potential for alteration of the pharmacodynamics of Fenestra® LC (pentobarbital).
c. Tail vein preparation: The tail vein was immersed in warm water for 30-60 seconds to increase blood flow to the tail and dilate the vessels prior to injection of Fenestra® LC. A similar effect may be achieved using an incandescent bulb.

Fenestra® LC Dosing

a. Route of administration: Fenestra® LC was injected intravenously into the lateral tail vein of the anesthetized mouse.
b. Size of dose: Fenestra® LC was administered at a dose of 0.4 mL per 20 g body weight. The dose can be adjusted for animal size, desired level of contrast enhancement, IACUC limitations on i.v. injection volume or study design.
c. Syringe size: A 1 mL disposable syringe fitted with a 30-ga needle is appropriate for injection of Fenestra® LC. Syringes from Terumo have been found to perform best of all syringes used during the development of this agent.
d. Injection rate: The 400 μL of Fenestra® LC was injected over a period of 30-60 seconds.
e. Saline flush: A saline flush is optional when using a syringe pump with an infusion set or catheter, especially in a larger animal, although caution should be used because Fenestra® LC is more dense than saline.
f. Expected residence time for imaging purposes: 0-6 hours post-injection, with peak at 3-4 hours post-injection
g. Dosing frequency may be at least every other day or longer; shorter dosing schedules may be possible but have not been evaluated by MediLumine.

Image Acquisition

a. Animal Orientation: The anesthetized mouse was placed on the imaging table in the prone position with its head oriented into the gantry. Use of anesthetic gas may require the mouse to be positioned with its tail toward the gantry. Upon obtaining the scout view, the desired body region was selected as the anatomic landmark for image acquisition. Wrapping the anesthetized mouse in a single layer of bubble wrap will help the animal maintain body heat during the scanning regimen.
b. Pre-contrast Exam: A pre-contrast scan of the anesthetized mouse may be acquired if desired as part of the study protocol.
c. Equipment Settings
    1) Image acquisition parameters for the MicroCAT II (ImTek, Inc., Knoxville, TN; distributed by Philips Medical, Cleveland, OH) are based on the type of study, subject size and desired spatial resolution. Imaging settings for this example of a medium resolution contrast-enhanced study were as follows:

(i) *X-ray Camera Setup*:

Parameter	***Setting***
Serial CCD length	2048
Parallel CCD length	3072
Serial Bin Factor	2
Parallel Bin Factor	2
Exposure Time	750 msec
Warp Correction	Yes
Defect Map Correction	No

(ii) *X-ray Tube Setup*: X-ray voltage 80.0 kVp; Anode Current 500.0 μA.

(iii) CT *Scan Setup*:

Parameter	Setting
Rotating Stage Start Position	0.000 degrees
Bed Axis Position	313.863 mm
Bed Height	43.026 mm
Detector Position	0.000 mm
Total Rotation	360 degrees
Number of Rotation Steps	520
Number of Axial Bed Steps	0
Number of Detector Steps	0
Number of Acquired Calibration Exposures	20
Projection Display Period	1
Raw Data	Written to File
Real Time Reconstruction	No
Total Scan Time	11.07 min

(iv) *Scanner Geometry Setup*:

Parameter	Setting
Source to Detector Distance	309.600 mm
Source to Center Distance	256.200 mm
Physical Detector Pitch	32.700 μm
Detector Array Height	2048 elements
Detector Array Width	3072 elements
Center Offset	4.7 unbinned detectors

2) Length of scan: The average time for image acquisition for hepatic imaging with Fenestra® LC was approximately 12 minutes for acquiring the scout view and the 520 step medium resolution study with the MicroCAT II.

d. Expectations: Images were obtained immediately after completion of injection, t=0, with subsequent scans acquired at t = 30, 60, 120, 180 and 240 min post-injection. At t = 0 and t= 30 min the vessels will have higher contrast levels than the liver, but by 60 min the liver will display similar levels of contrast enhancement. At later time points the vessels will become hypodense relative to the liver which will maintain a relatively stable enhancement through 4-6 hours post-injection. Hepatic tumors will display very little or no contrast enhancement during the duration of the study. Hepatobiliary elimination of Fenestra causes liver contrast values return to baseline levels within 18-24 hours post-injection.

Data Reconstruction

a. Reconstruction Parameters

Machine-based reconstruction of the contrast-enhanced liver images obtained in this example does not allow for down-sampling of the projections prior to reconstruction as does a software-based reconstruction program. Down-sampling was unnecessary for the selected resolution in this instance. The reconstructed image file is stored as a .ct or .att file, which can be exported to

Amira for viewing as axial, coronal and sagittal images in addition to a number of other image representations.

Parameter	*Setting*
Number of Voxels in Volume	512 x 512 (transaxial) x 768 (axial)
Voxel Size	100 μm (transaxial) x 150 μm (axial)
Reconstruction Filter	Shepp-Logan
Reconstruction Algorithm	Feldkamp cone-beam

Data Visualization

a. Amira 3.1: Data is routinely imported from the ImTek reconstruction program as raw CT image data or as bitmaps windowed to a vascular contrast setting determined by the operator. Data can be viewed in Amira 3.1 (TGS, Inc.) using the Standard Display format with simultaneous display of the axial, coronal and sagittal images, as a 3D isosurface image which can be manipulated to view more or less anatomic structure with or without Orthoslice display of 1, 2 or all 3 of the planar slices. The isosurface image may also be cropped to eliminate extraneous data and saved as an Amira map file, which speeds isosurface viewing and saves storage space.

b. Exporting images: Planar and 3D images can be captured for presentations or publication purposes utilizing the Amira image capture feature. Movies can be created for fly-through of 3D image data sets.

Representative Images

Representative images from studies conducted in CT-26 tumor-bearing male mice using Fenestra® LC as described in this User Guide are provided in the figures below.

Precontrast Exam

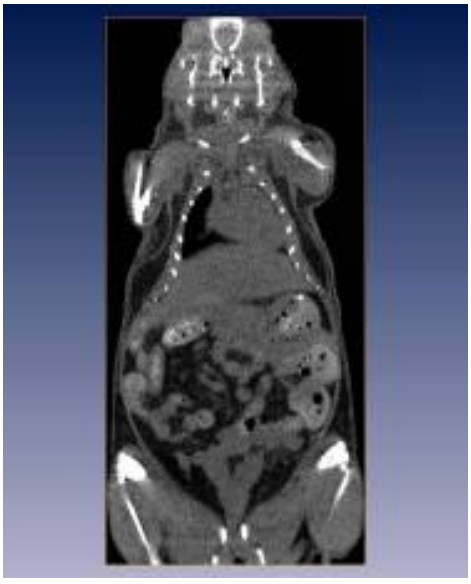

Figure 1. Non-contrast coronal scan of male mouse obtained with MicroCAT II. Poor soft tissue contrast is evident in the thoracic and abdominal cavities. The bright spots observed in the intestines are caused by minerals in the rodent chow that attenuate X-ray energy.

Fenestra® LC Exam

Post-contrast Images

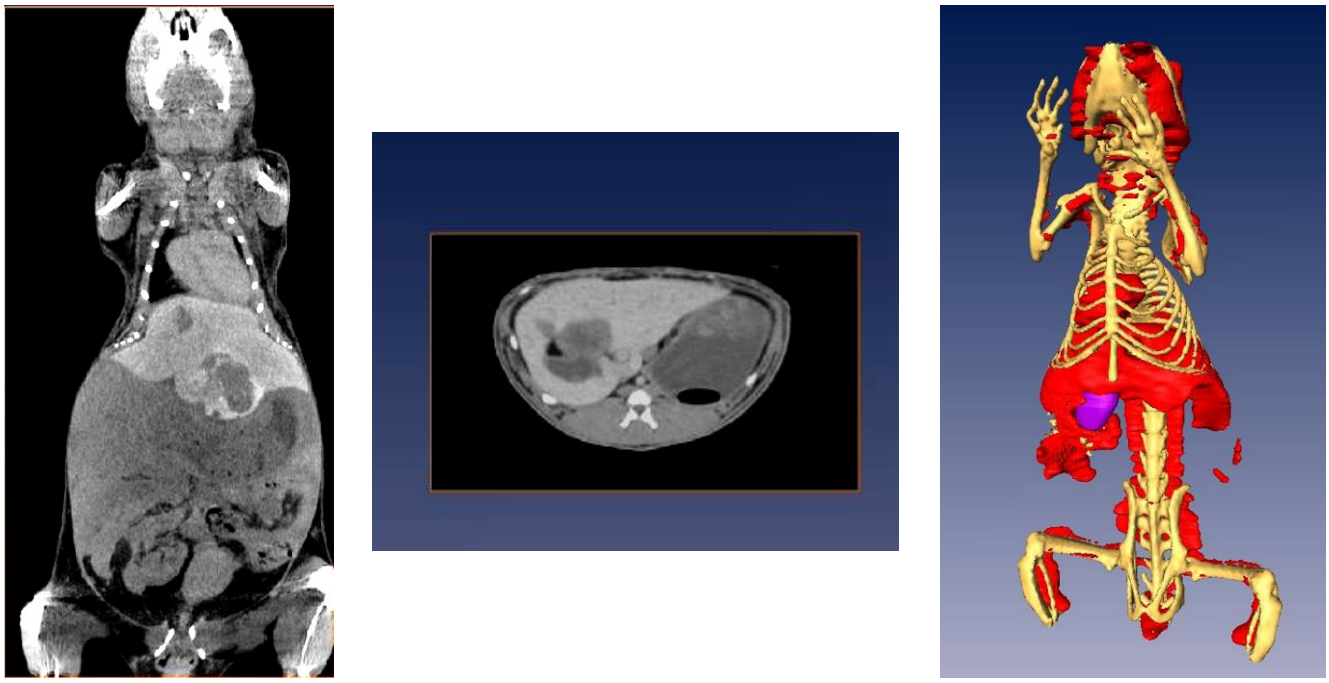

Figure 2. Coronal view (left image), transverse view (middle image) and 3D reconstruction (right image) of male Balb/c mouse with hepatic CT-26 adenocarcinoma 2 hr after IV injection of Fenestra® LC. At this time point the vascular system is still enhanced as evidenced by the lack of hepatic vascular detail and isodensity of the ventricles of the heart. The hypodense gall bladder near the dome of the liver is readily visualized in the coronal view. The CT-26 tumor is observed at the lower edge of the liver with a narrow rim of enhanced liver below the tumor and was colorized (purple) in the 3D reconstruction. The lack of artifact in the lower abdomen is a result of the mouse having been fed a soft, non-chow diet for 48 hr prior to imaging.

Troubleshooting

Why didn't I get good vascular contrast?

There are a number of reasons why the contrast enhancement and image quality for any given study will vary. The most common source for poor contrast enhancement is complete or partial extravasation of the dose during injection. The easiest way to tell if your injection was successful is to scan the tail of the animal – if the tail is bright, the injection was extravasated. Other possible explanations for poor contrast enhancement include inappropriate imaging settings (see section above on Image Acquisition), too low of a dose (0.3-0.4 mL/20 gm mouse is best), inappropriate windowing levels during data visualization, unsuitable reconstruction algorithms, inappropriate anesthesia (animal too light can cause considerable motion artifacts; certain anesthetics cause periodic gasping which also creates motion artifact) or imaging too late (agent has cleared) after injection.

When should I be imaging?

When using Fenestra® LC in mice, optimal liver contrast enhancement is provided at approximately 4 hours after injection of the dose, but earlier time points can provide perfectly adequate contrast enhancement as well. For other species, optimal timing will be different. Contact technical support for experience in other animal species.

Why is there so much image artifact?

Inappropriate selection of anesthesia or plane of anesthesia induced within an animal can have a dramatic effect of image artifact. Inhalation anesthesia normally results in the animal having greater respiratory motion than if an injectable anesthetic (many of which induce respiratory depression) is utilized. Injectable anesthetics, however, may introduce other undesirable effects ranging from periodic respiratory gasping, altered metabolism of Fenestra LC (or other test article present for the study), or susceptibility to overdose death. Inadequate anesthetic dosing will yield significant motion artifact due to respiratory motion regardless of the chosen anesthetic.

Contents in the gastrointestinal tract can also introduce significant artifact. Most commercial laboratory animal chows contain considerable quantities of radiopaque minerals that cause significant image artifacts. If possible, fast animals prior to the conduct of imaging studies or, preferably, place the animals on a liquid or soft vegetable diet for 24-48 hrs prior to the imaging study.

How big a dose can I give?

Doses as high as 20 mL/kg have been well tolerated in preliminary studies conducted using normal mice. It is important, however, to check with your institutional IACUC regarding dose volume limitations for each species.

Technical Support contact information:

MediLumine Inc.
5795 av. de Gaspé, Suite #112, CTS Healthcare Campus
Montreal (Quebec), Canada, H2S 2X3

Tel: 514.360.1574 ı US Toll free: 1.844.360.1574
Fax: 514.360.6014

medilumine.com ı info@medilumine.com