Spectrum CT 202
Advanced Imaging of Fluorescent and Bioluminescent Probes

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Advanced Imaging Training Manager
Day 1

10:00 – 12:00 AM  Presentation – Advanced Imaging of Fluorescent and Bioluminescent Probes
IVIS Spectrum and Living Image Software
Imaging Concepts
Optical Imaging Workflow and Experimental Design
Advanced Fluorescence – Epi vs. Transillumination and Spectral Unmixing
3D Tomography

12:00 – 1:00 PM  Lunch

1:00 – 5:00 PM  Hands on with Phantom Mice
Basic Acquisition Overview with focus on BLI
Tool Palette In-depth
Bioluminescence Tomography

Day 2

9:00 – 12:00 PM  Hands on with Phantom Mice
Fluorescence Acquisition
Spectral Unmixing
Transillumination with Fluorescence Tomography

12:00 – End  Wrap up – Live animal imaging if provided
Why Optical In Vivo Imaging?

- Wide range of applications
- Useful for functional imaging and tracer applications
- Reduce number of animals
- Simple instrumentation, interface, and integration into current workflow
- Amount of light is proportional to number of copies of the gene or amount of fluorescent probe

Bioluminescence features:
- Unmatched sensitivity
- Highest Signal:Noise
- Easiest analysis

Fluorescence features:
- Brighter signal
- Multitude of reporters
- Translational
- Ease of use
- High sensitivity CCD for bioluminescence or fluorescence imaging
- High throughput (23 cm) or high resolution FOV
- 28 discrete bandpass filters 490 – 850 nm
- Monochromatic imaging or spectral unmixing
- Reflection (Epi-) or transmission-mode fluorescence
- 3D optical tomographic reconstructions for both bioluminescence and fluorescence
- Ideal for imaging multiple probes/reporters
- Low-dose µCT x-ray exposure for longitudinal studies
- High resolution, smaller field of view µCT for detailed analysis
Living Image® Software

- Controls all settings in the IVIS® system (fully computer controlled)
- Advanced cataloging and browsing tools
- Analysis tools for quantification
- Instrument settings are analogous to photography
- User-friendly interface
- Imaging Wizard assists in choosing optimal setup and analysis parameters
- Auto-exposure for optimal image capture settings
- microCT acquisition setup and reconstruction is simplified and optimized for ease-of-use
Camera and Lens Settings are Analogous to Photography

- Field of View (FOV) is dependent on the distance from the lens to the sample
- Light collected is proportional to how long the shutter is open (exposure time)
- Aperture (f/stop) controls the amount of light collected
- Digital pixel binning is possible on the CCD – alters sensitivity/resolution
Setting Sensitivity – Signal Level

- The IVIS® CCD camera has a raw signal range of 0 to 65,535 Analog to Digital counts ($2^{16}$ or 16-bit)

- Adjust camera settings to obtain a signal level of 600 to 60,000 counts

- Settings that control signal level are:
  - Exposure time
  - Pixel binning (CCD resolution)
  - $f$/stop (aperture)

- Instrument is calibrated to automatically compensate for changes in sensitivity settings
Exposure Time

- Signal level is directly proportional to exposure time (1:1)
- Shorter exposure time improves throughput
- Recommended minimum exposure time > 0.5 seconds
- Longer exposure times increase signal intensity
- Recommended maximum exposure time < 5 minutes
**f/stop (Lens Aperture)**

- **f/stop** controls the amount of light received by the CCD detector.
- **f/1** is wide open, maximum light collection – default for luminescent.
- **f/8** is smallest aperture, best resolution – default for photo.
- Changing f/stop changes counts by a factor of 4.

![f/stop Diagram]
Pixel Binning (CCD Resolution)

- Binning refers to the grouping of pixels into a larger super-pixel
- Changing binning settings changes counts by a factor of 4

- Large Binning (16 x 16)
  Higher Sensitivity/Lower Resolution
- Medium Binning (8 x 8)
  Default
- Small Binning (4 x 4)
  Lower Sensitivity/Higher Resolution
Calibrated Physical Units

- Living Image® automatically compensates for device settings: Exposure time, f/stop, binning and field of view.
- Calibrated unit is Radiance, representing the flux radiating omni-directionally from a user-defined region.

2 sec exposure, f/stop 1, Small binning
~5000 counts peak
2.82 x 10^8 photons/sec

10 sec exposure, f/stop 1, Small binning
~25000 counts peak
2.82 x 10^8 photons/sec
Calibrated Physical Units vs. Raw Signal

Raw Signal

(Counts)

Exp time: 30 sec 30 sec 60 sec 60 sec 60 sec 60 sec
Binning: small small small small medium medium
Day: 1 2 3 4 5 6

Peak Counts

1600 1200 800 400
Calibrated Physical Units vs. Raw Signal

**Calibrated Signal**

(Photons per second)

**Exp time:**
- 30 sec
- 30 sec
- 60 sec
- 60 sec
- 60 sec
- 60 sec

**Binning:**
- small
- small
- small
- small
- medium
- medium

**Day:**
- 1
- 2
- 3
- 4
- 5
- 6

**Radiance:**

Photons per second
Measurement Table

- Measurement table displays information about each Region of Interest (ROI)
- Table is user-configurable and can be exported to a spreadsheet
**Reporter Molecules**

- Luciferase
  - + ATP and O$_2$ – Live cells
- Fluorescent Proteins
  - + D-luciferin substrate
- Quantum dots and Nanoparticles
- Fluorescent dyes
  - IVIS XenoLight DiR

**Transfection**
- Genetic Marker

**Direct cell/protein labeling**
- Label Cells
- Label Bacteria
- Label Proteins

**Excitation light source**
Visualization 21 days before palpation

Bioware Ultra

- Data collection from Day 0
- Tumors too small for physical measurement until 21+ days
Dual Reporter: Bacterial luc and GFAP Brain Imaging

Simultaneous visualization of:

- *Streptococcus pneumonia* (lux)
- Glial Fibrillary Acidic Protein - GFAP (luc)

Kadurugamuwa *et al.*, *Inf. And Immun.* 2005
Dual Bioluminescence and Fluorescence for Molecular Profiling

Orthotopic 4T1 Tumors

White Light  Bioluminescence  Epifluorescence

4T1 Bone Metastasis

Bioluminescence  Epifluorescence

For assistance: April Blodgett (508) 589 7461 or April.Blodgett@perkinelmer.com
Ex vivo Analysis for Validation

Transgene upregulation in brain
Atenuation of Bioluminescent Probes

Experimental Design

Optimal Imaging Window
Luciferin Kinetics Critical for Quantitative Reliability and Reproducibility

- Intraperitoneal (i.p.) Injection
- Immediate availability
- Need to wait for uniform substrate distribution
- Optimal imaging window where > 95% of max signal intensity observed
- Plateaus typically 10-20 minutes in duration

Diagram:
- Substrate Distribution Highly Variable
- Uniform Distribution
- Substrate Clearance Highly Variable
- Plateau > 95% Max 10-20 min

Graph:
- Y-axis: Percentage Maximum Intensity
- X-axis: Time (min) Post Substrate Injection
- Immediate availability
- Optimal Imaging Window
- Substrate Clearance Highly Variable
- Substrate Distribution Highly Variable

Diffuse Light Imaging Tomography
Challenges of Fluorescence Imaging – Diffusion, Scattering AND Autofluorescence

- Excitation light needed for fluorescent sources
- Light traveling through tissue scatters many times creating a "fuzzy" image at the surface of the animal
- The IVIS® views the diffuse image on the surface of the subject
- With fluorescent sources, the diffusion pattern is overlaid with autofluorescent signal from skin

Experimental Design
Attenuation of Fluorescent Probes

Experimental Design

Optimal Imaging Window

Hemoglobin $\mu_a$ (cm$^{-1}$)

Normalized Intensity

Wavelength (nm)

- HbO2
- GFP Excitation
- GFP Emission
- mCherry Excitation
- mCherry Emission
- XenoLightCF 680 Excitation
- XenoLightCF 680 Emission
- XenoLightCF 750 Excitation
- XenoLight CF 750 Emission
Fluorophore Spectra and Autofluorescence

Experimental Design
Animal Diet Autofluorescence

- Alfalfa detection highest between 660 and 740
- Many 645 and 680 nm probes overlap
- Low autofluorescence chow helpful if working in this range
- Research Diets
  http://www.researchdiets.com
  AIN-76A (D10001i) – alfalfa free
- 750 probes allow one to bypass these issues as well
Depilation – Important Regardless of Modality

Fur removal via depilation increases intensity for BOTH bioluminescent and fluorescent models

- All fur attenuates
- Dark fur most attenuating
- Shaving prior to depilation is preferred
- Caustic - Do not leave on mouse skin for extended periods!
- Repeat periodically when new hair growth is visible
Positioning – Important Regardless of Modality

- Determine optimal positioning
- Keep positioning consistent for most accurate quantitative data
**Sensitivity is a Function of a Signal to Noise**

**Luminescent Sources:**
Signal brightness generally lower than fluorescent sources

Higher sensitivity due to low level noise: both instrument and animal autoluminescence

**Fluorescence Sources:**
Signals generally brighter than luminescent sources

Lower sensitivity due to higher noise: instrument background and autofluorescence
Improvements to Signal to Noise Ratio

**Adaptive FL Background Subtraction:**
Software tool to reduce instrument background

**Spectral Unmixing:**
Extracts fluorescent signal from autofluorescence
IVIS® Spectrum CT Fluorescence Components

- CCD, TE-cooled to -90°C
- Lens assembly
- Emission filter wheel
- Excitation filter wheel
- Optical switch
- Heated sample stage
- Transillumination Fiber bundle
- 10 excitation filters
- 18 emission filters

Advanced Fluorescence Imaging
Fluorescence Acquisition

Advanced Fluorescence Imaging
Fluorescent Calibrated Units: Radiant Efficiency

Compensates for non-uniform excitation light pattern

Radiant Efficiency = \frac{\text{Emission Light (photons/sec/cm}^2/\text{str)}}{\text{Excitation Light (}\mu\text{W/cm}^2)}
What is Spectral Unmixing?

- Simply put – separating colors
- Pixel by pixel analysis that allows for distinguishing components in an image based on wavelength

Why use Spectral Unmixing?

- Increase signal to noise when high levels of autofluorescence are present
- Specifically separate co-localized probes
How is Spectral Unmixing accomplished?

- Images acquired at multiple wavelengths
- Pixels mapped and grouped based on peak
- Pick emission filters centered around component peaks
Spectral Unmixing – Removing Autofluorescence

Normalized Intensity

Wavelength (nm)

535nm Excitation Filter

Autofluorescence Excitation

tdTomato Excitation

580nm Emission Filter

Spectral Unmixing – Removing Autofluorescence

Advanced Fluorescence Imaging
Spectral Unmixing – Removing Autofluorescence

Tissue AF

Normalized Amplitude

Wavelength (nm)

S:N = 1.5

S:N = 4053

Advanced Fluorescence Imaging
Spectral Unmixing - Modes

Automatic

- Traditional Caliper method
- Fully automated
- Fluorophore spectral libraries pre-loaded
- Easiest but least exact

Compute Pure Spectra

- Traditional CRi method
- Manual control by user
- Spectral libraries created by user
- Uses a type of image math to subtract autofluorescence from “mixed components”

- Three modes:
  - Manual
  - Guided
  - Library

- More input required but typically better outcome – higher S:N
Spectral Unmixing - Library Generation

- Effective spectral unmixing requires good library
- Library gives the software the spectral information for individual components of the image
- User generated thereby tailored for your experiment
- Effective library generation begins with inclusion of proper controls
- **Autofluorescence control** critical, use either:
  - Naïve animal handled same way
  - Diseased with no probe injected
- **Nonspecific control** elucidates sites of probe clearance
- For experiments involving simultaneous injection of more than one probe, have one control for each probe to be used
Spectral Unmixing – Library Generation

- Manual Unmixing
- Mark pixels to generate average curve
- Label appropriately
- CPS – Image Math to subtract autofluorescence component and generate Pure components
- Unmix
Why Perform Spectral Unmixing? Quantitation

Advanced Fluorescence Imaging

**Monochrome: Total Signal vs. Concentration**

- $R^2 = 0.7095$

**Unmixed: Total Signal vs. Concentration**

- $R^2 = 0.9117$
Why Perform Spectral Unmixing?  **Multiplexing**

- Unmixing prevents crosstalk between far and near infrared reporters

- Allows visualization of colocalization and accurate quantitation *in vivo, in vitro or ex vivo*

Shcherbakova, DM and Verkhusha VV., *Nat. Met.* 2013
- Optimized for imaging deep signals, improves Signal:Noise ratio
- Avoids problems from whole body autofluorescence created from Epi-illumination

Select desired locations in Living Image for Raster scan

Selected excitation positions denoted by crosshairs in acquired image
Normalized Transmission Fluorescence

Fluorescence
- Filter pair specific for probe
- Standard wizard setup

Transmission
- Neutral density filter
- White light
- Tissue optical density
- No user input required
- Automatic when Normalized checked – default for 2D transillumination

NTF Efficiency is unit of measure for 2D transillumination
- Negative exponent – large denominator
Epi vs. Transillumination Imaging of XL680, XL750 and QD800

Transillumination optimal for deep tissue imaging – greater sensitivity at depth

- **CF680 dye**
  - 250 picomole
  - S/B: 0.81 (EPI), 6.69 (NTF)

- **CF750 dye**
  - 250 picomole
  - S/B: 1.11 (EPI), 9.19 (NTF)

- **QD800 dye**
  - 4 picomole
  - S/B: 1.31 (EPI), 8.32 (NTF)

- **CF680 dye**
  - 25 picomole
  - S/B: 1.18 (EPI), 2.47 (NTF)

- **CF750 dye**
  - 25 picomole
  - S/B: 1.11 (EPI), 4.27 (NTF)

- **QD800 dye**
  - 0.4 picomole
  - S/B: 1.05 (EPI), 2.57 (NTF)

Detect 0.4 picomole Qdots in the lungs
Benefits of 3D Imaging – Why I Need It?

2D

Flexible, fast, easy!!!

Over 6000 publications using FLI and BLI in vivo, ex vivo, and in vitro in numerous models

Throughput

Quantitation

Colocalization

Coregistration

Calibrated to NIST standard – 2D quantitation is cornerstone of most IVIS publications!

HOWEVER, transillumination facilitates detection of probes in deep tissue

Calibration curves translate radiance units into pmol or cells

Algorithms account for diffusion, scattering and attenuation

Ability to answer more complex biological questions

Anatomical context allows for pinpoint localization

Localization from 2D imaging difficult without ex vivo confirmation

Due to diffusion, 2D surface radiance is less exact when determining localization of probes

3D reconstruction can elucidate location of multiple probes in relation to each other


Li, Chunsheng, et al. Oncotarget 2014


Quantification – Attenuation Correction

- Changing depth impacts surface radiance more intensely
- Algorithms account for red shifting due to attenuation
- Resultant values at times more indicative of actual in vivo outcome

- “2D BLI imaging … 80% reduction in average radiance and 71.8% reduction in total flux”
- “3D DLIT …. 20.2% reduction in total flux, a 16.7% decrease in source volume, and a 1.5 mm increase in source depth (8.3 to 9.8 mm”
- “Gross pathology confirmed a medial necrotic zone … lateral area of grossly viable tissue”
- “Microscopic pathology confirmed that approximately 14.9% of the total tumor area demonstrated morphologic evidence of coagulative necrosis.”
Quantification

Experimental Design

- GastroSense 750 serially diluted and orally gavaged into nude mice
- Epi vs Transillumination/FLIT performed for detection and quantification.
- Transillumination provides consistent quantitative analysis throughout the range of dilutions
- Epi-illumination values consistently 2-3 fold lower than expected
- 3D results show accurate location and volumetric approximation even with decreasing intensity

Source Perkin Elmer Technical Note - Optical Imaging on the IVIS Spectrum CT System: General and Technical Considerations for 2D and 3D Imaging
Quantification – Transillumination Facilitates Deep Tissue Detection and 3D

Experimental Design

- 1 million 4T1-rLuc injected directly into lungs 2 weeks prior
- Integrisense 750 injected 24hrs prior to imaging

- Epi-illumination not capable of distinguishing IntegriSense 750 from background in lungs
- Transillumination facilitates both detection and 3D analysis

- Heavy tumor burden in lungs around 40 million viable cells causing decrease in air volume visible via μCT
- Around 2 nmol Integrisense 750 detected in lung cavity specifically in areas of heavy tumor burden as indicated by colocalized signal
- αvβ3 integrin upregulation in tumor cells indicating growth, viability, and angiogenesis

<table>
<thead>
<tr>
<th>Reporter</th>
<th>3D ROI</th>
<th>Total Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firefly Luc</td>
<td>ROI 1</td>
<td>4.07E+07</td>
</tr>
<tr>
<td>IntegriSense 750</td>
<td>ROI 1</td>
<td>2.34E+03</td>
</tr>
</tbody>
</table>

Unpublished results – PerkinElmer
Colocalization

- 2D surface radiance is impacted by scattering and diffusion.
- Determinations about colocalization at depth purely based on 2D is risky especially in deeper tissue.
- 3D colocalization can aide in confirming colocalization at depth.

- "confirm the specific localization of 78Fc to hTEM1-positive grafts"
- "bioluminescent and NIR fluorescence signals overlapped only at the site of hTEM1-positive grafts”
- "fluorescence was observed in the livers of these mice”
- "demonstrated that 78Fc750 was specifically enriched in hTEM1-positive tissues.”
- "observed a localized tumor mass in the cortex”
- "GFAP activity was upregulated more broadly in the areas surrounding the tumor….In agreement with the immunohistochemical data”
- "suggesting that the GFAP response in the luciferase reporter extends beyond the immediate tumor margin”
Coregistration – Pinpoint Localization/Anatomical Context

- Anatomical context to optical reconstruction
- Pinpoint localization

- UPEC inoculated into the bladder and ascend the ureter to cause pyelonephritis
- 3D identified localization in intestinal tract after ingestion

- Day 13
- Day 20

Front View of Urinary Tract

- Kidney
- Ureter
- Bladder
- Sphincter
- Urethra

3D Optical Tomography
2D surface radiance is impacted by scattering/diffusion/attenuation.

- Exact determinations about location purely based on 2D is impossible *in vivo* in deeper tissue areas.

- 2D provides no specific information about brain region

3D provides depth and MRI coregistration confirms pinpoint localization of tumor in brain

Unpublished data courtesy Dr. Adrienne Scheck, Barrow Neurological Institute, Phoenix, AZ

- "GAP-43 signals were indeed arising from the areas surrounding the brain structures normally affected by MCAO, including parts of the frontal and parietal cortex, striatum, and hippocampus."

- "These findings were further validated by immunohistochemistry analysis."

- "The measured tumor depths ranged from 1.2 mm to 6.8 mm"

- "At the sites of tumor metastases, osteolytic lesions were evident by μCT imaging"

- "co-registered the 3D bioluminescent images with 3D μCT images to display all metastatic lesions in a referenced anatomical setting."
Multimodal image coregistration and inducible selective cell ablation to evaluate imaging ligands


Coregistration – Anatomical Context

- “Peak luminescence levels within the large intestine region, correlating with results obtained by sampling and culturing.”
- “The proximity … the pancreas prevented the accurate delineation of a ROI encompassing solely the pancreas on PET images.”
- “An ROI comprising only the pancreas (red dashed line in Fig. 1 D and E) was defined by using the tomographic bioluminescence image”
- “allowed for unambiguous delineation of the pancreas ROI in … PET image … enabled us to quantify the pancreatic PET signal while minimizing the confounding influence …. signal from adjacent organs.
- “facilitated the anatomical positioning of bioluminescent signal sources within the tumour”
- “in addition to visualisation of tumour vasculature through administration of a contrast agent.”
- “UCC2003 was detected solely in necrotic regions, as evidenced by PCR. 3D…imaging provides in vivo evidence of this here”
Single View 3D Imaging is a Two-Step Process

3D bioluminescence imaging: Diffuse Light Imaging Tomography (DLIT)

The optical camera acquires a series of 2D luminescence images with different emission filters, ranging from 560-640 nm.

CT generates animal surface for DLIT reconstruction.

CT X-ray source

Emission filter wheel with multiple filters

X-ray Detectors

3D trans-illuminating fluorescence imaging: Fluorescence Imaging Tomography (FLIT)

The optical camera acquires a series of 2D fluorescence images with the excitation light source at different positions.

CT generates animal surface for FLIT reconstruction.

CT X-ray source

Emission filter

Excitation filter

Light source (Position adjustable)

X-ray Detectors

3D Optical Tomography
Surface Topography Reconstruction – Step 1

- CT scan needed
- Full body CT scan
- Fast or Standard Resolution option
- Use threshold presets to determine edges of subject
DLIT - Spectral Measurements Provide Information on Depth of Source

In vitro

In vivo

Subcutaneous

In vivo

Chest Cavity
Luciferin Kinetics Critical for DLIT

- Remember! Consistent light output assumed for algorithmic reconstruction

- All 5 images need to be acquired in this timeframe

- Careful of factors that may affect curves – localization, handling, metabolism

Burgos et al., 2003

Bioluminescence Tomography
### DLIT Reconstruction

- Select tissue properties
- Select source spectrum
- Select wavelengths
- Threshold your data

### Data Table

<table>
<thead>
<tr>
<th>Chest Cavity</th>
<th>Peritoneal Cavity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth [mm]</td>
<td>Flux [photons/sec]</td>
</tr>
<tr>
<td>2.1</td>
<td>$2.43 \times 10^8$</td>
</tr>
<tr>
<td>Depth [mm]</td>
<td>Flux [photons/sec]</td>
</tr>
<tr>
<td>3.2</td>
<td>$1.44 \times 10^8$</td>
</tr>
</tbody>
</table>
Automatic Mouse Atlas Registration

- Anatomical localization in Spectrum

3D Tomography
Transillumination Facilitates 3D Reconstructions of Fluorescent Sources

Subject

Stage

Excitation Light Source

Select desired locations in Living Image

Selected excitation positions denoted by crosshairs in acquired image

Kidneys

Kidneys

Fluorescence Tomography
The excitation source scans the subject

The CCD collects multiple projections using designated emission filter

Angle and intensity of projections facilitates reconstruction of source localization and intensity
FLIT Reconstruction

- Select tissue properties
- Threshold your data

• Select images
Reagents and Instrumentation - 3D Tomographic Analysis

For assistance: April Blodgett (508) 589 7461 or April.Blodgett@perkinelmer.com
Bacterial Targeting of Tumors – Dual Reporter 3D Tomography

HTC116 Cancer Signal

Brevibacterium Bacterial Signal

Dual Cancer/Bacterial Signal

Mark Tangney and Cormac Gahan, University of Cork

Cronin et al., PLOS One, 2012
Injectable probes offer valuable insights into the molecular mechanisms involved in disease progression – necrosis, angiogenesis, apoptosis, hypoxia, tissue viability.

2D epillumination analysis allows visualization of necrosis in the liver and bladder compared to control.

However, 3D tomography facilitated by deep tissue detection capabilities of transillumination elucidates deeper AnnexinVivo signals and facilitates compartmentalization of signals.

Anatomical context provides information about pinpoint location of probes and tomography allows quantitative analysis of probe concentrations in deeper tissues.

In contrast to 2D, 3D allows simultaneous monitoring of necrosis and clearance of the probe through the kidneys.
Use Well Plate Quantification Library to Determine pMol or Cell Number

- Dilute your cells or dye and image
- Select Well Plate Quantification from Tools menu
- Choose library when reconstructing
- Enter cell number or concentration per well
- Save as a library
Absolute Quantification and Anatomical Reference

1 million 4T1-rLuc injected directly into lungs 2 weeks prior

Integrisense 750 injected 24hrs prior to imaging

Diseased lungs show decrease in lung air volume via µCT

Heavy tumor burden in lungs around 40 million viable cells causing decrease in air volume

Around 2 nmol Integrisense 750 detected in lung cavity specifically in areas of heavy tumor burden as indicated by colocalized signal

αvβ3 integrin upregulation in tumor cells indicating growth, viability, and angiogenesis

<table>
<thead>
<tr>
<th>Sequence Number</th>
<th>3D ROI</th>
<th>Voxels</th>
<th>Total Cells</th>
<th>Average Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRE20130626193512_SEQ</td>
<td>ROI 1</td>
<td>10684</td>
<td>4.07E+07</td>
<td>3.81E+03</td>
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<tr>
<td>BRE20130626195138_SEQ</td>
<td>ROI 1</td>
<td>14025</td>
<td>2.34E+03</td>
<td>1.67E-01</td>
</tr>
</tbody>
</table>

Bioluminescence signal expressed in # cells

Fluorescence signal expressed in pmol

3D Overlay BLI, FLI, Colocalized
Micro-Computed Tomography (µCT)

- Provides anatomical reference and structural measurements
- Calibrated measurement in Hounsfield units
- Utilized to reconstruct surface for DLIT and FLIT
- Can generate 2D X-ray projection onto 2D bioluminescent or fluorescent images
- User friendly setup and analysis
How µCT Works

Voltage = 50 kV
Current = 1 mA

Higher tissue density = Higher attenuation
µCT Analysis and Units

- Attenuation = 1/Penetration
- Increasing photon energy generally decreases attenuation
- Higher energy photons are more penetrating
- Calibrated automatically for Hounsfield unit scale.
- Linear transformation of the original linear attenuation coefficient measurement
  - Radiodensity of distilled water at STP is 0
  - Radiodensity of air at STP is -1000
  - Density of tissue dictates HU

**HU** = 1000 × \( \frac{\mu_x - \mu_{\text{water}}}{\mu_{\text{water}}} \)
## Scan Options with µCT

<table>
<thead>
<tr>
<th>Mode Name</th>
<th>Resolution (µm)</th>
<th>Voxel Size (µm)</th>
<th>FOV L x W x H (cm)</th>
<th>Binning</th>
<th>Dose (mGy)</th>
<th>Scan Time</th>
<th>Typical Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast</td>
<td>850</td>
<td>300</td>
<td>12.5 x 12.5 x 3</td>
<td>4</td>
<td>13</td>
<td>3.6</td>
<td>Needed for FLIT/DLIT, longitudinal CT</td>
</tr>
<tr>
<td>Standard (1 Mouse)</td>
<td>425</td>
<td>150</td>
<td>12.5 x 12.5 x 3</td>
<td>4</td>
<td>53</td>
<td>14.4</td>
<td>Anatomical reference, largest FOV</td>
</tr>
<tr>
<td>Standard (2 Mice)</td>
<td>425</td>
<td>150</td>
<td>12.5 x 12.5 x 3</td>
<td>4</td>
<td>23</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Medium Res</td>
<td>225</td>
<td>75</td>
<td>6 x 6 x 3</td>
<td>2</td>
<td>132</td>
<td>36</td>
<td>Best for soft tissue, organ contrast</td>
</tr>
<tr>
<td>High Res</td>
<td>150</td>
<td>40</td>
<td>2.4 x 2.4 x 2</td>
<td>1</td>
<td>46</td>
<td>72</td>
<td>Bone detail, implant study</td>
</tr>
<tr>
<td>High Res Top</td>
<td>150</td>
<td>40</td>
<td>2.4 x 2.4 x 2</td>
<td>1</td>
<td>46</td>
<td>72</td>
<td>Bone detail, implant study</td>
</tr>
</tbody>
</table>

### Limitations of X-ray Dosage

- Immuno-compromising Dose = 1000-2000 mGy
- LD 50/30 Dose = 5000-7500 mGy
Resolution

Turn X-ray on with the provided key

When armed, the Acquire button will display this message

Best for use in CT analysis

Optimal for soft tissue contrast

Highest resolution CT only

Best for anatomical reference

Highest
225 μm

Standard
450 μm

Fast
850 μm
μCT Analysis – Multimodality Module Overview

- 3D ROI for Hounsfield Unit
- Render 2D X-ray view for overlay onto bioluminescent or fluorescent images
- Crop and adjust histogram to segment based on density
Visualization Tools

- Data can be cropped to remove unwanted regions or to slice into animal for visualization purposes.

Before cropping | Crop on X | Crop on Y | Crop on Z

Multimodality - Software
Visualization Tools

- Histogram shows distribution of voxels from least to most dense – lowest to highest HU
- Maps can be generated to isolate voxels of a particular density, saved and reused
- Used to isolate tissues and/or contrast agents

Bone and Exitron/Vasculature
Bone
Bone and Lung
Visualization Tools

- Data can be displayed in two ways:
  - Gradient – Enhances boundaries between homogeneous regions
  - Maximum Intensity Projection (MIP) – Projects maximum intensity voxels in viewing plane
Visualization Tools

- 3D animation tools for generating videos
- Multiple video formats available for export
- Slice tab allows for visualization of multiple slices simultaneously
- Raw data mode or Volume Color Table mode if you wish to see segmented components only
Visualization Tools

- Three main groups of tissue can be imaged with CT:
  - Bone
  - Fat
  - Soft tissue
- Lungs can typically be visualized due to the presence of air within the organ
- Utilizing heat maps may improve visualization of tissues when compared to grey scale
For proper data analysis, datasets should be normalized to a common scale bar.
Utilizing newly included tools, common settings can be applied across a group of images.
View of the images can be synced as well.
Utilize mouse atlas as further confirmation of organ location when lacking contrast
For an In-Depth Study

IVIS Software Manual

IVIS University Web page
www.perkinelmer.com
In Vitro Bioluminescence, In Vivo Bioluminescence, In Vitro Fluorescence, In Vivo Fluorescence

Optical and Micro-computed Tomography (CT) on the IVIS Platform
Top Ten Tips for Optical Imaging

1. Choose reporters that maximize signal-to-noise (S:N) ratio
2. Consider the appropriate control groups and imaging time points necessary
3. Use hairless mice or white-furred animals and depilate or shave
4. Switch to autofluorescence-free mouse diet
5. Closely map the kinetics of your biological bioluminescent model
6. Animal handling can significantly affect kinetics
7. Image in the animal orientation that yields the highest signal intensity
8. Cover intense signal to allow dimmer signals to dictate auto-exposure
9. Utilize guards to prevent reflection off neighboring animals
10. Use black well plates when doing in vitro experimentation

Thank you for your attention!

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