



In-Vivo Imaging: **IVIS Spectrum**

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Advanced Imaging Training Manager

What will be covered?

Introduction

- Principles of Optical 'In Vivo' Imaging
- Key IVIS[®] Hardware components
- Overview of Living Image[®] Software
- Fluorescence Options

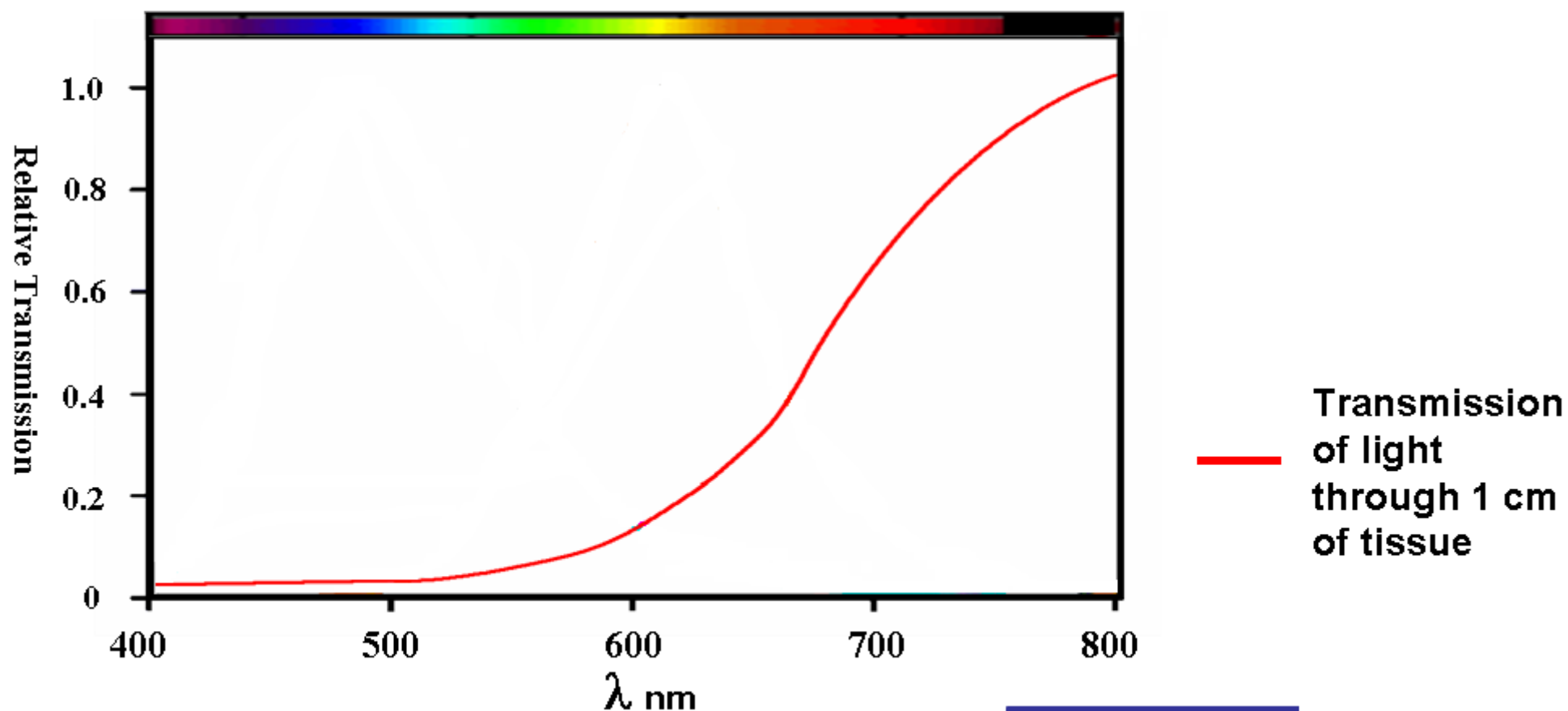
Training

- Hands on Training

Why Optical *In Vivo* Imaging?

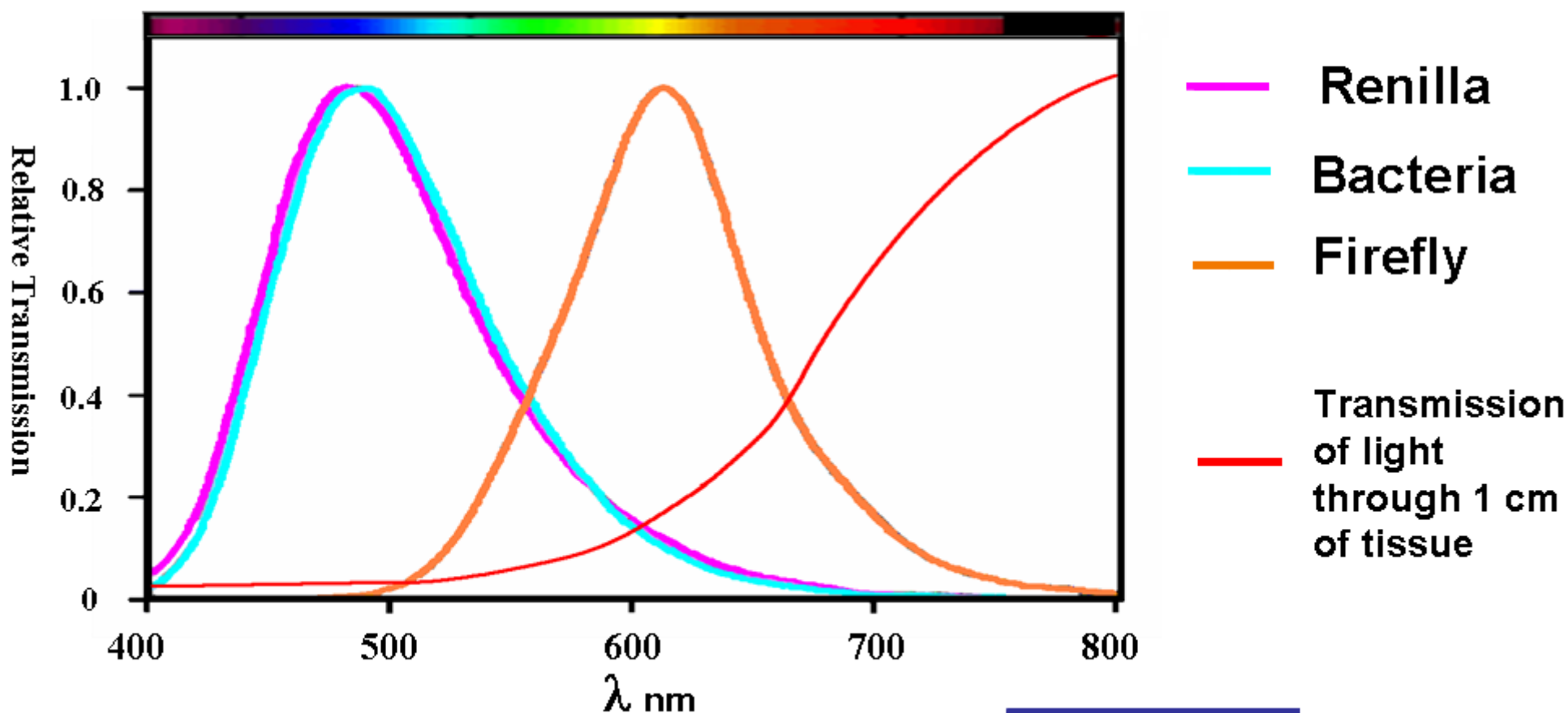
- Powerful labeling technique - gene expression results in production of luciferase
 - Amount of light is proportional to number of active live cells
 - Typical applications range from oncology studies, infectious disease (tracer) to imaging transgenic animals (functional)
- Non-invasive – does not require subject to be euthanized
- Relatively simple instrumentation.

Tissue is not Transparent - Light Absorbance Depends on Wavelength

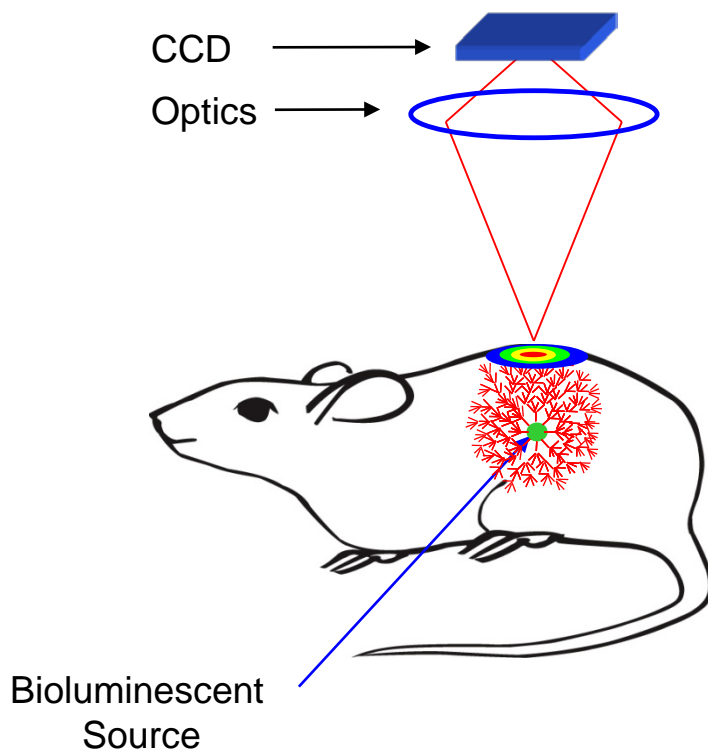


Tissue is not Transparent - Light Absorbance Depends on Wavelength

Luciferase Emission Spectra and Tissue Transmission

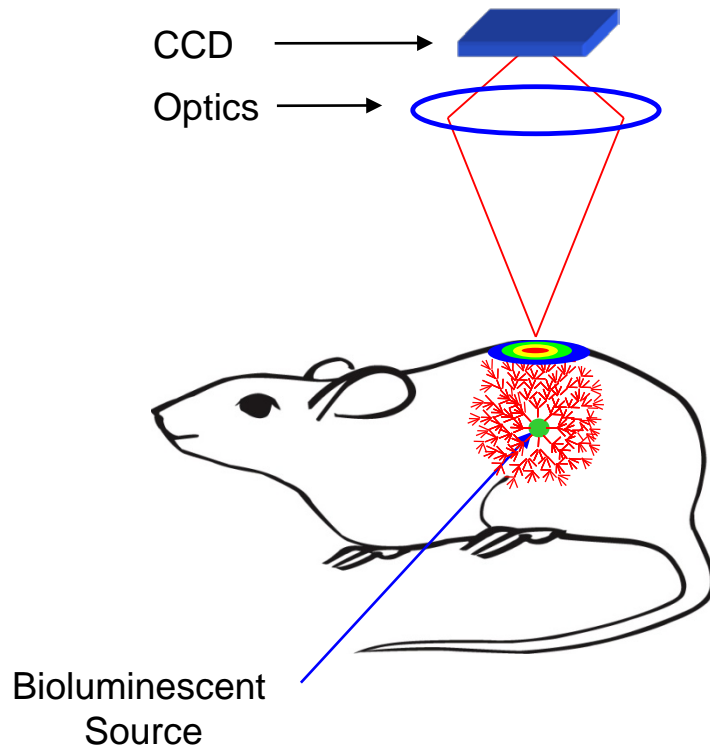


Photons Diffuse Through Tissue and the IVIS[®] Views this Signal on the Surface of the Subject

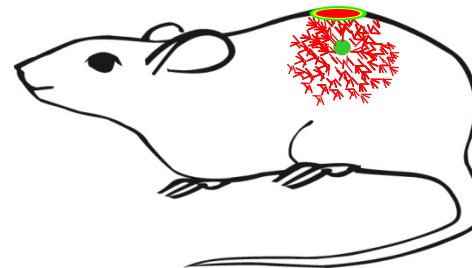


- Light traveling through tissue scatters many times creating a "fuzzy" image at the surface of the animal
- The IVIS[®] views the diffuse image on the surface of the subject

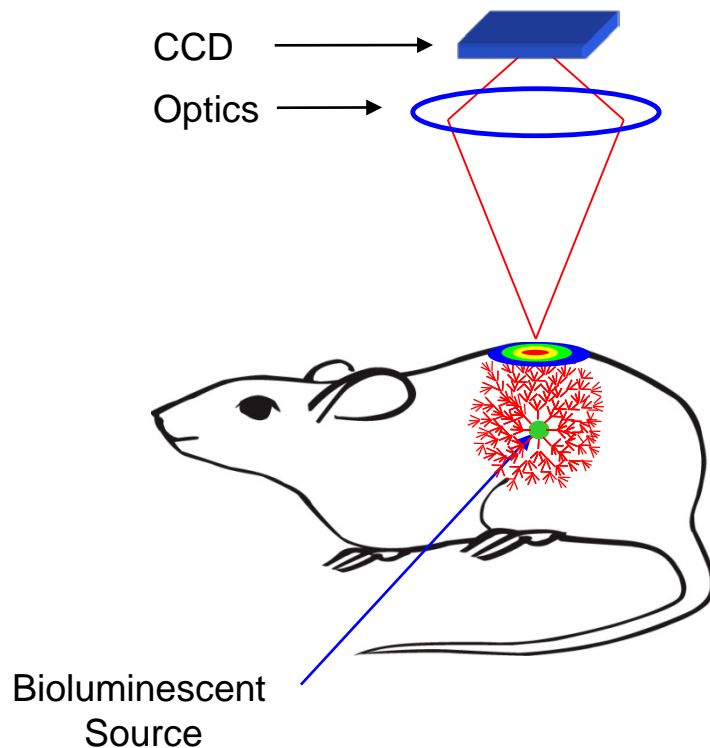
Photons Diffuse Through Tissue and the IVIS[®] Views this Signal on the Surface of the Subject



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Photons Diffuse Through Tissue and the IVIS[®] Views this Signal on the Surface of the Subject



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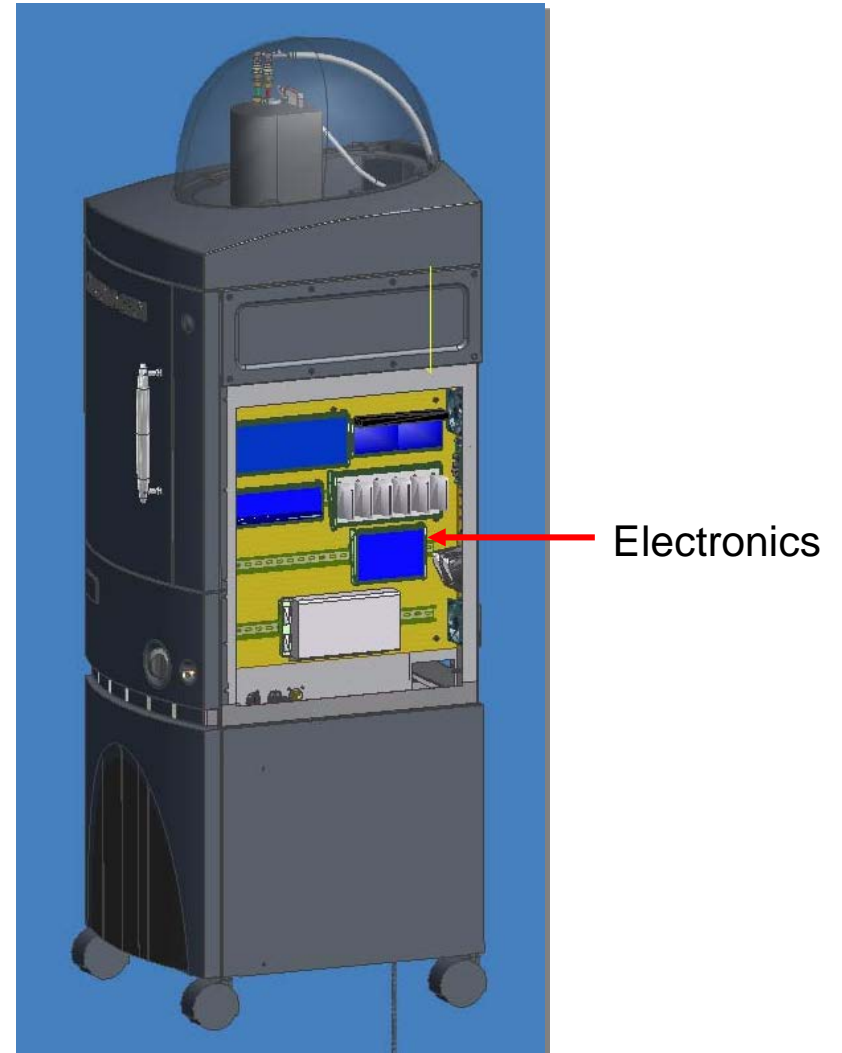
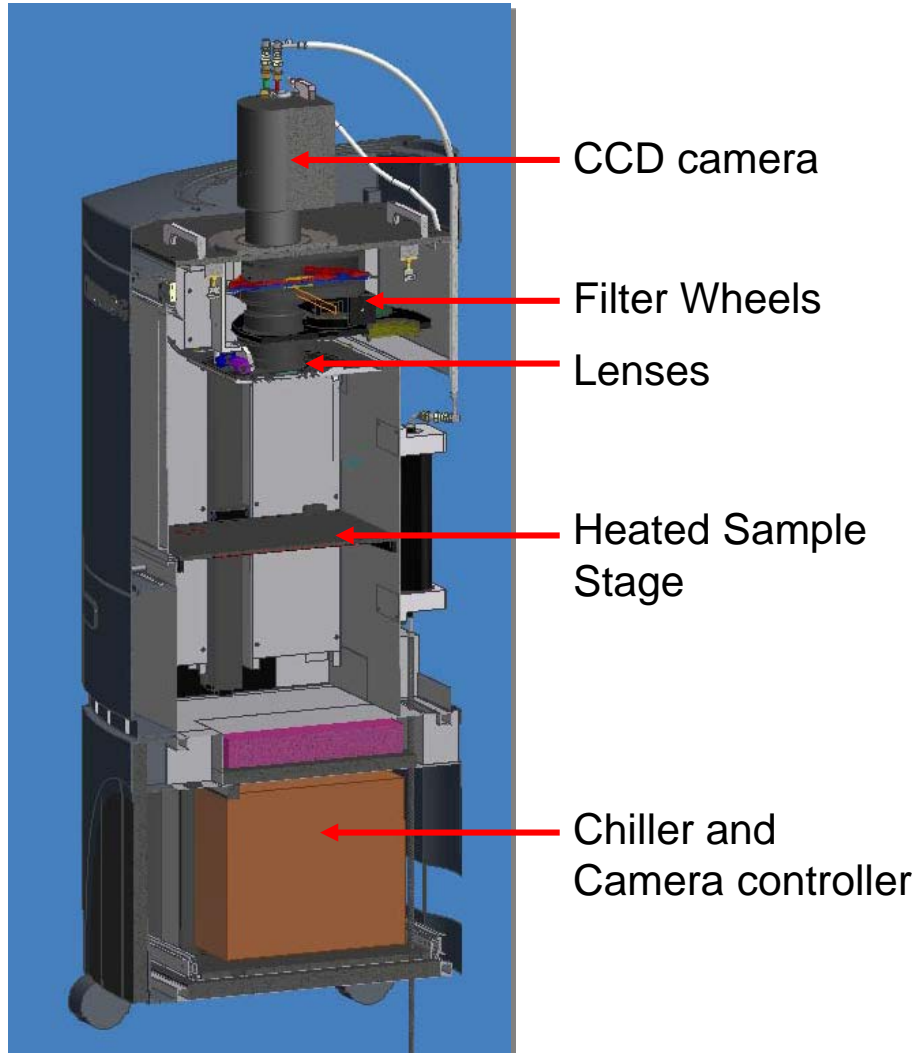


IVIS[®] Spectrum Imaging System - Hardware



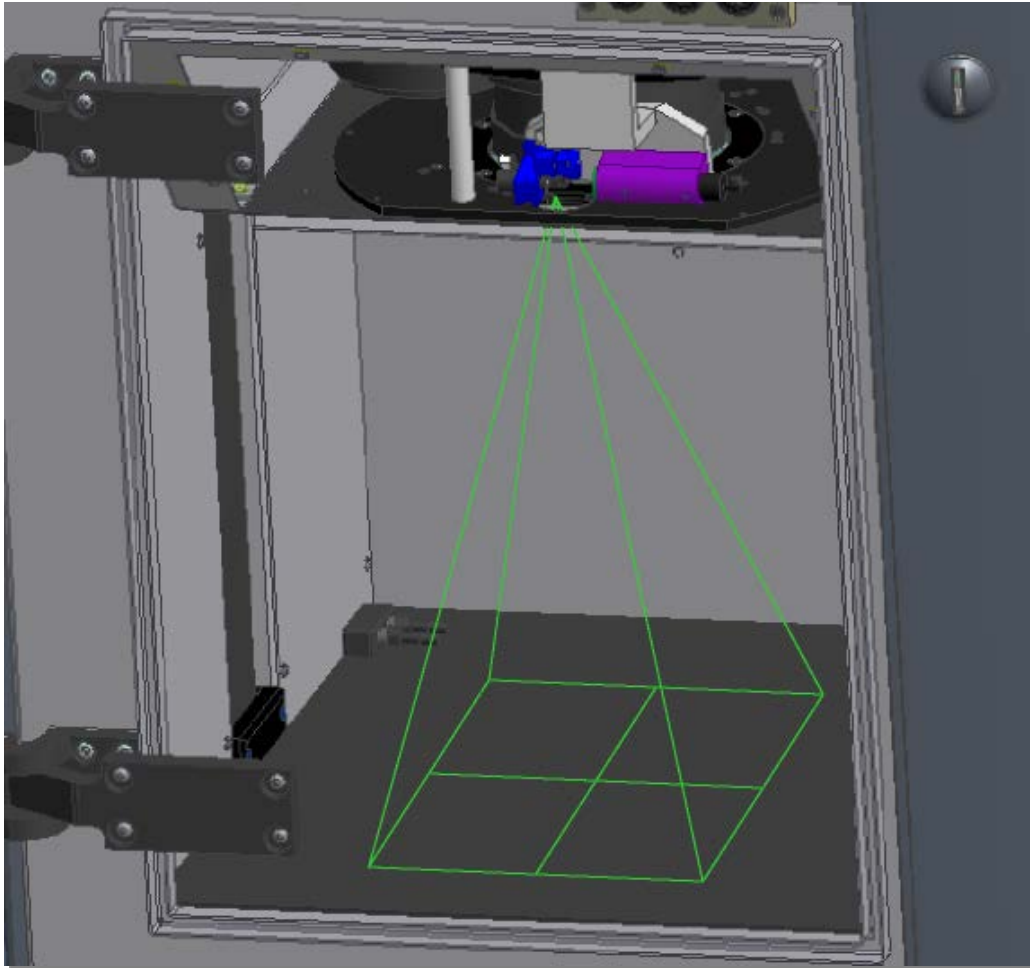
- Customized for *in vivo* imaging
- Highly sensitive camera with a large dynamic range

IVIS[®] Spectrum Imaging System – Hardware

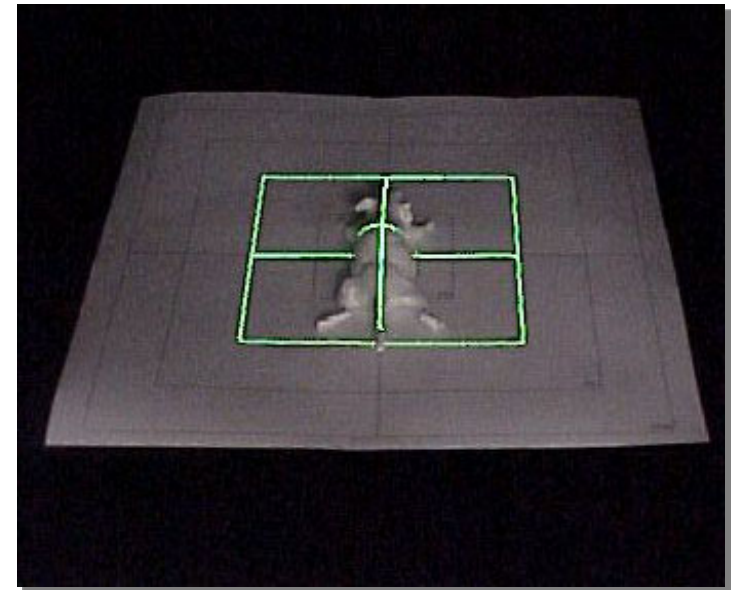


Hardware

Alignment Light Projector



- Allows rapid and reproducible positioning of subjects.
- Size changes with Field of View setting

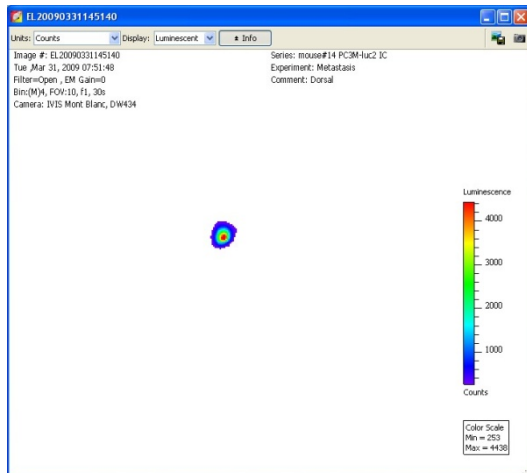


Hardware

Living Image[®] Software

- Controls all settings in the IVIS[®] system (fully computer controlled)
- Provides advanced cataloging and browsing tools
- Provides analysis tools for quantification
- Instrument settings are analogous to photography
- Images are acquired in a two step process

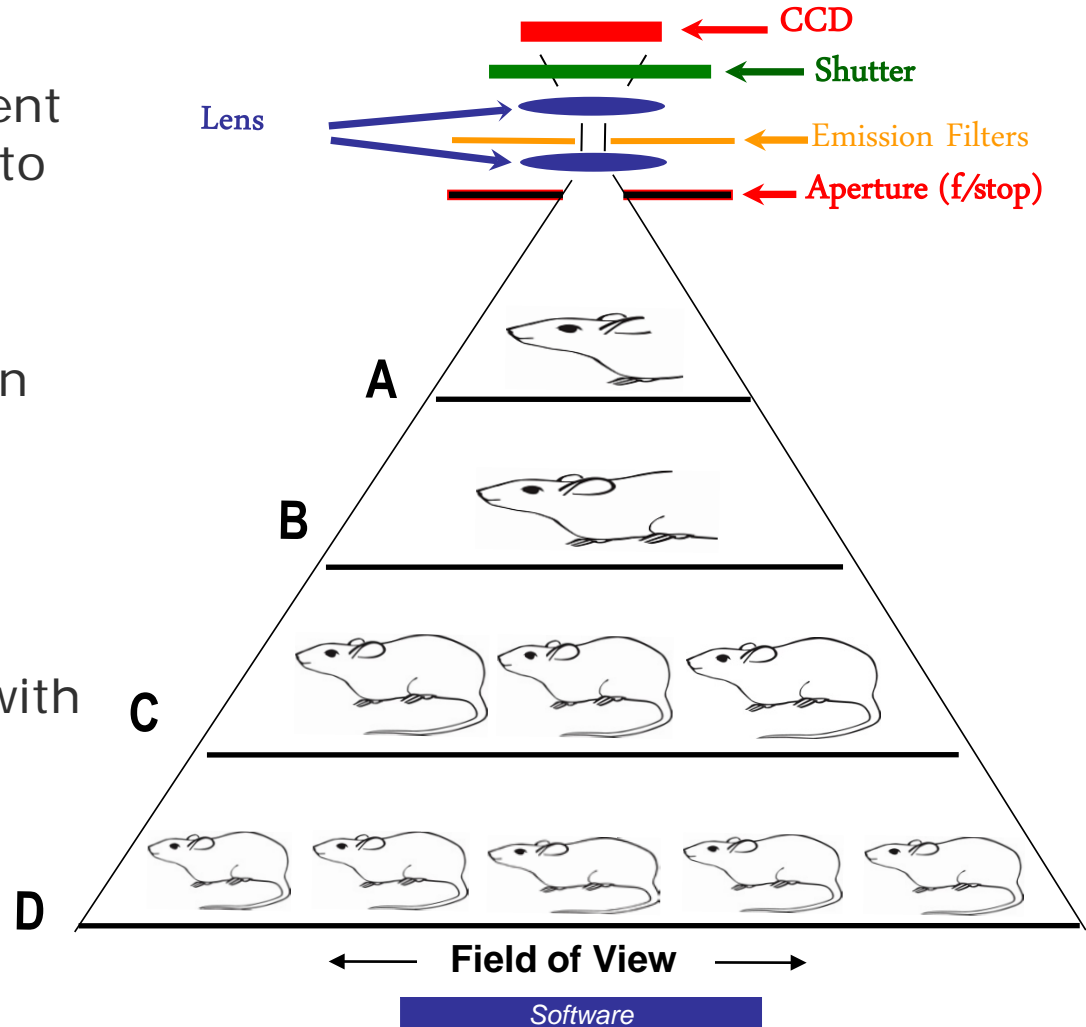
Standard Images are Composed of Two Images Photographic + Luminescent = Overlay



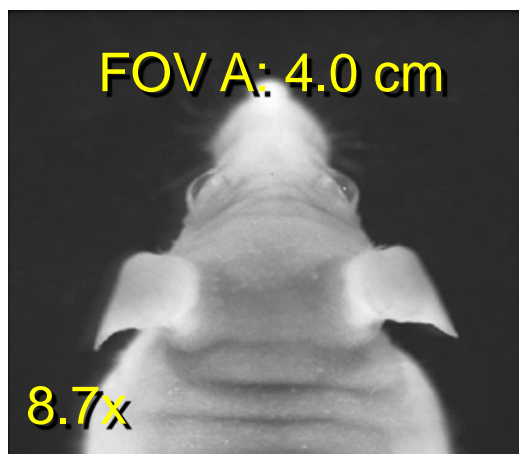
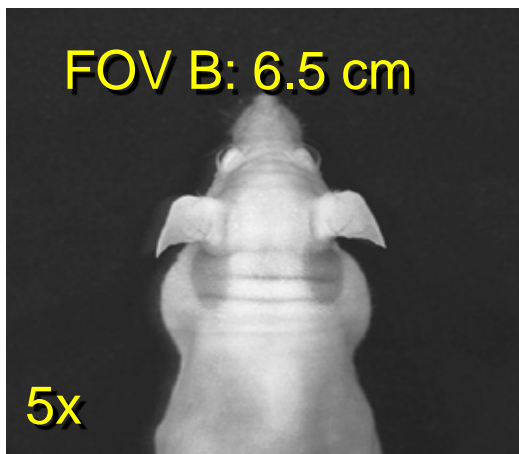
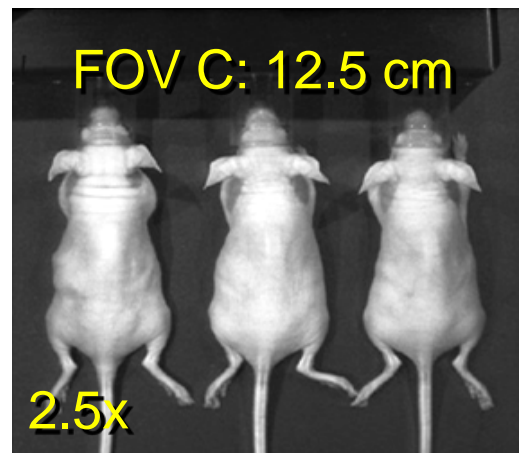
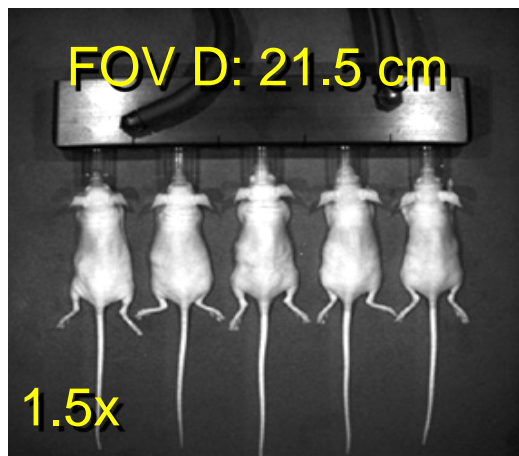
Software – Acquisition

Camera and Lens Settings are Analogous to Those Used in Standard 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 possible with CCD - for further increase in sensitivity



Field-of-View (typical)



Setting Sensitivity – Luminescent Signal Level

- The IVIS[®] CCD camera has a raw signal range of 0 to 65535 Analog to Digital Counts (2^{16}).
- Adjust camera settings to obtain a signal level of 600 to 60,000 counts.
- Settings that control signal level are:
 - Exposure time
 - Binning (CCD Resolution)
 - f/stop (Aperture)
- Instrument is calibrated to automatically compensate for changes in sensitivity settings

Living Image Control Panel

Controls Sensitivity

IVIS Acquisition Control Panel

Imaging Mode: Luminescent, Fluorescent, Photograph, Structure

Exposure Time: 1.00 sec, Binning: Medium, F/Stop: 1

Excitation Filter: Block, Emission Filter: Open

Field of View: C, Service: 12.9 cm, Subject height: 1.50 cm, Focus: use subject height

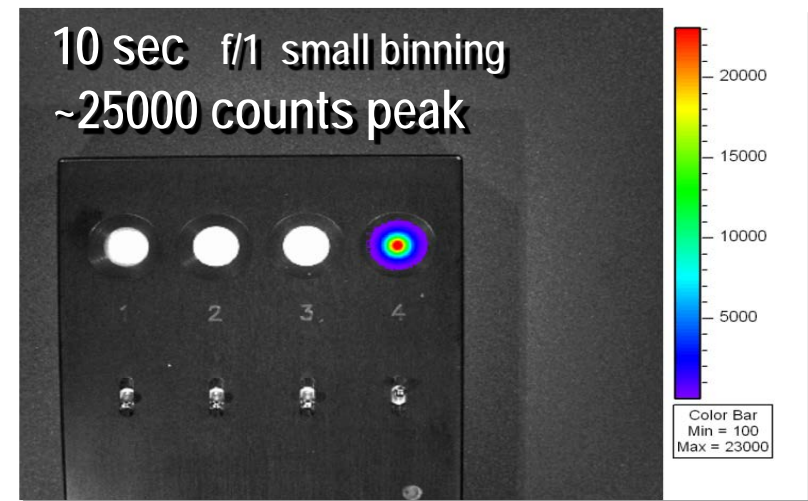
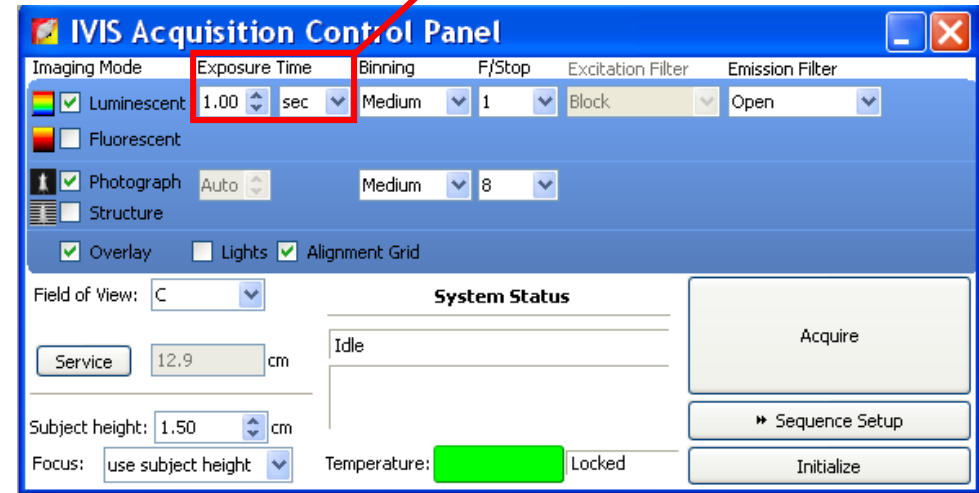
System Status: Idle, Temperature: [Redacted] Locked

Buttons: Acquire, Sequence Setup, Initialize

Options: Overlay, Lights, Alignment Grid

Exposure Time

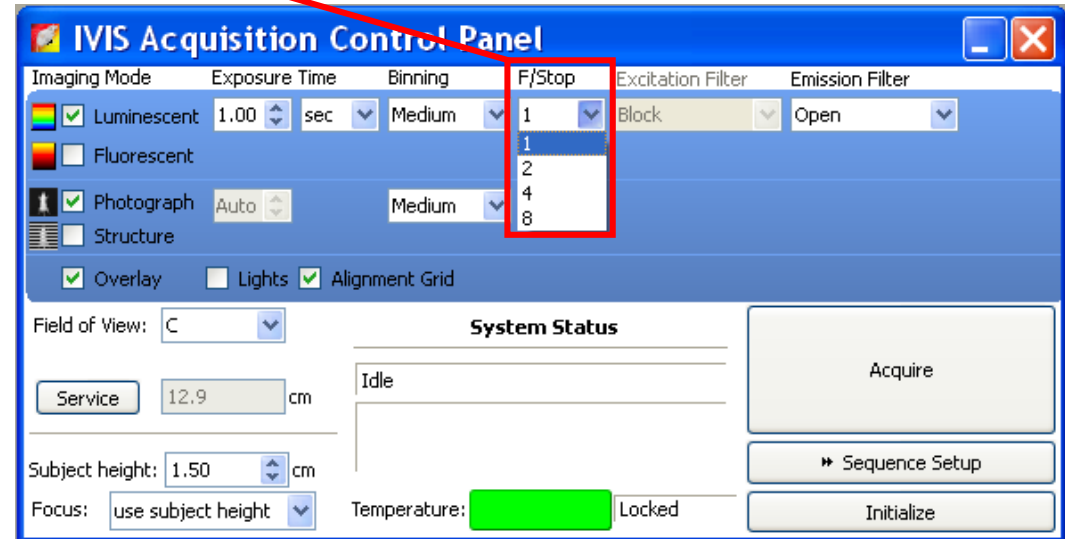
- Signal level is directly proportional to exposure time
- Shorter exposure time improves throughput
{ Recommended min exposure time > 0.5 secs }
- Longer exposure time increases signal
{ Recommended max exposure time < 5 mins }



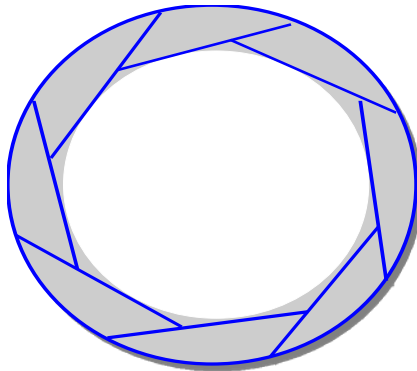
Software – Acquisition

f/stop (lens aperture)

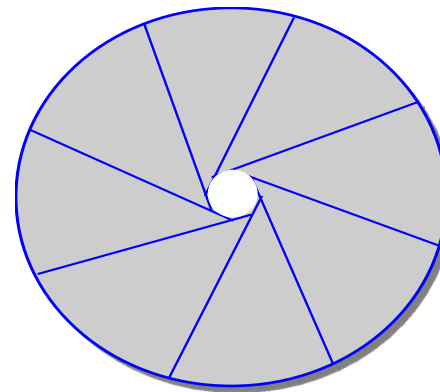
- f/stop controls the amount of light received by the CCD
- f/1 is wide open, maximum light collection - default for luminescent
- f/8 is smallest aperture, best resolution - default for photo



f/1



f/8

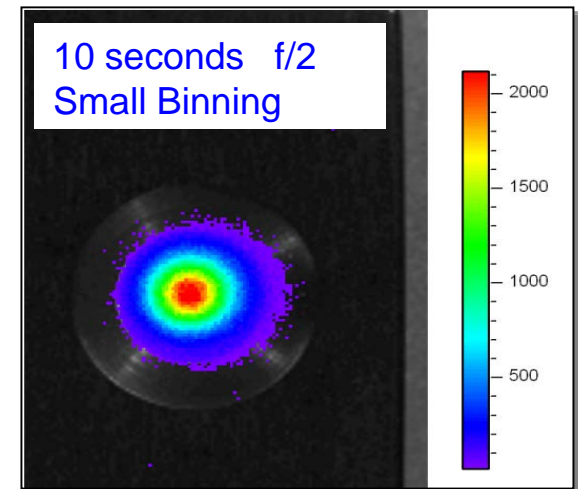
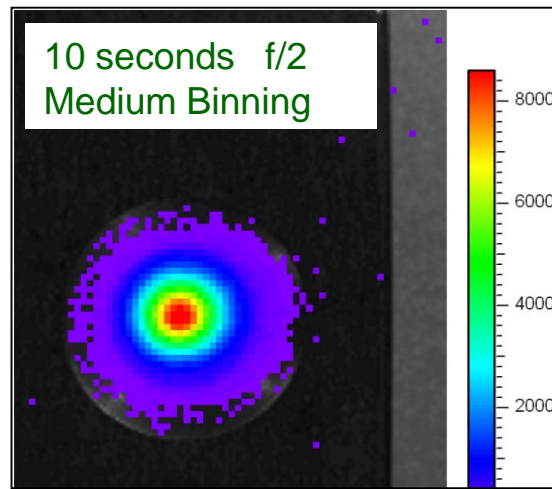
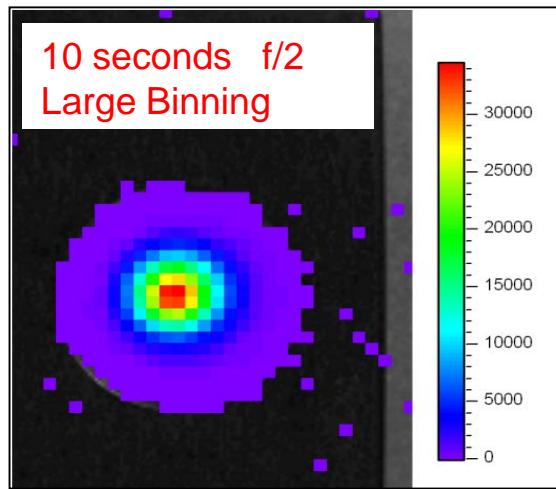
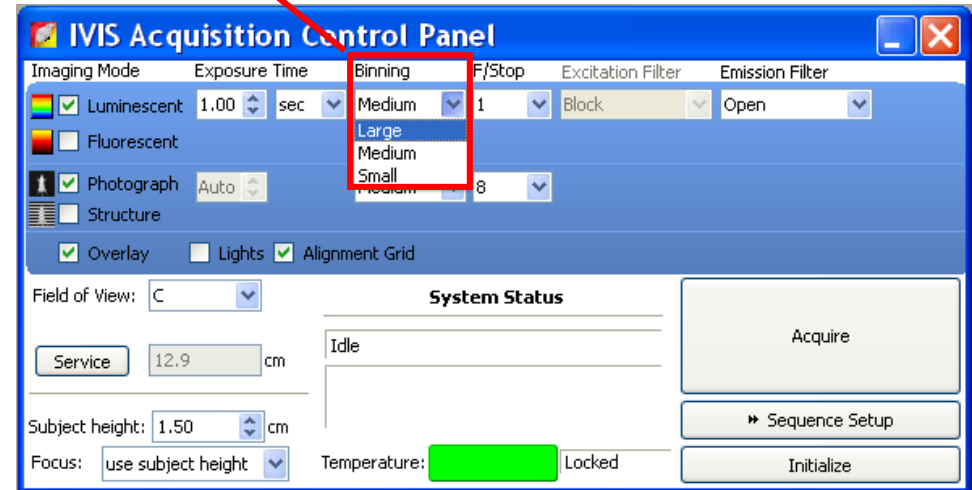
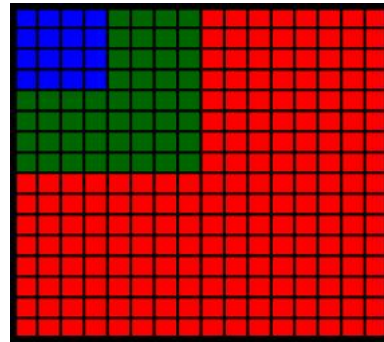


Software – Acquisition

Pixel Binning (CCD Resolution)

Binning refers to the grouping of pixels into a larger super-pixel

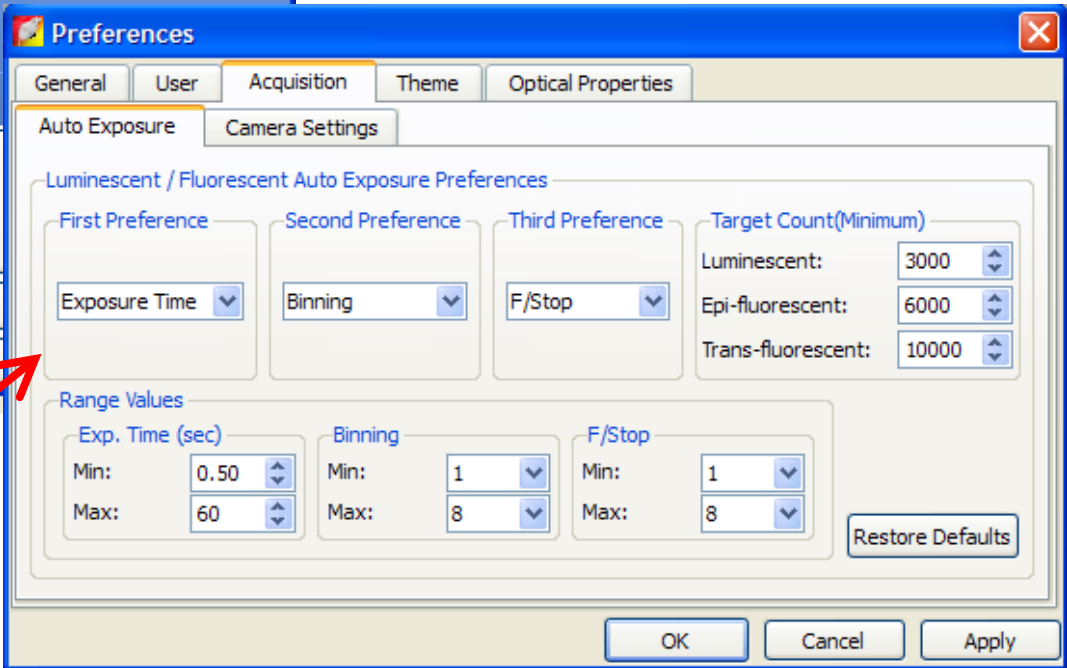
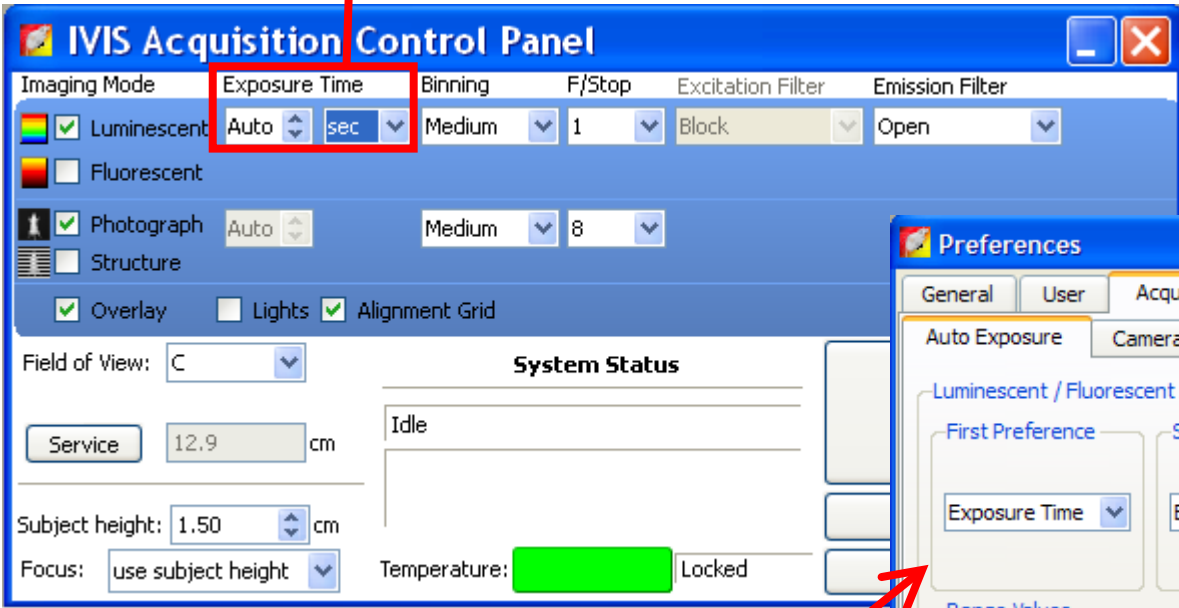
- **Large Binning (16)**
Higher Sensitivity/
Lower Resolution
- **Medium Binning (8)**
- **Small Binning (4)**
Higher Resolution /
Lower Sensitivity



Software – Acquisition

Auto-exposure

Auto-exposure feature available for bioluminescence and fluorescence



User definable settings

Summary of Basic Camera Settings

Controls Sensitivity

IVIS Acquisition Control Panel

Imaging Mode: Luminescent Fluorescent

Exposure Time: 1.00 sec Binning: Medium F/Stop: 1

Excitation Filter: Block Emission Filter: Open

Photograph (Auto) Structure

Overlay Lights Alignment Grid

Field of View: C

Service: 12.9 cm

Subject height: 1.50 cm

Focus: use subject height

System Status

Idle

Temperature: [redacted] Locked

Acquire

Sequence Setup

Initialize

Acquisition – Single Image

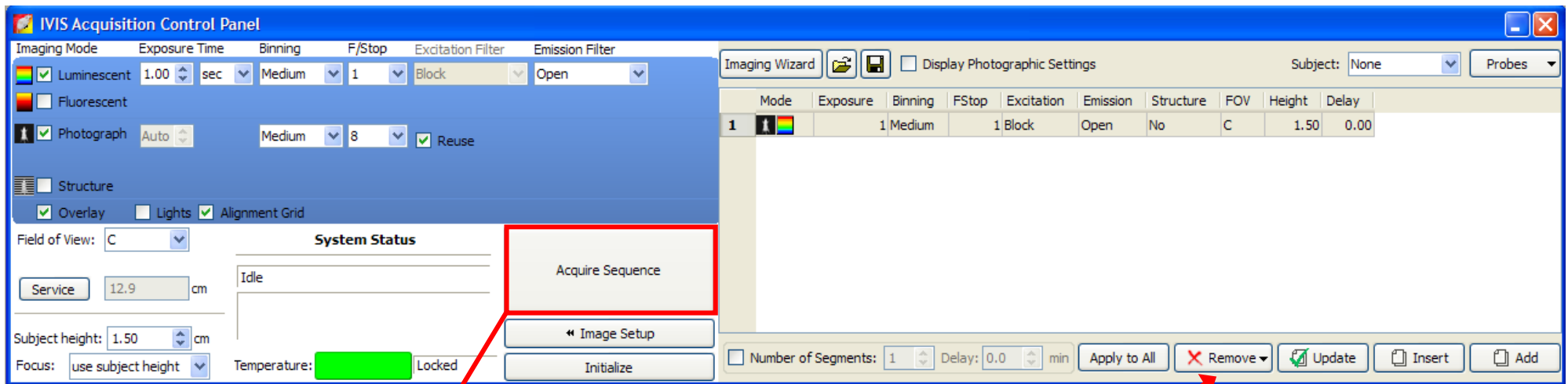
Overlay will automatically take Photo + Luminescent

Single Image Acquisition

Acquisition – Sequential mode

Allows automatic acquisition of a series of images separated by fixed time points.

(useful option for kinetic studies and DLIT 3D reconstruction)



Starts
Sequential
Image
Acquisition

User Friendly
Sequence Editor

Imaging Wizard

Select for assistance in setting up bioluminescence or fluorescence sequences

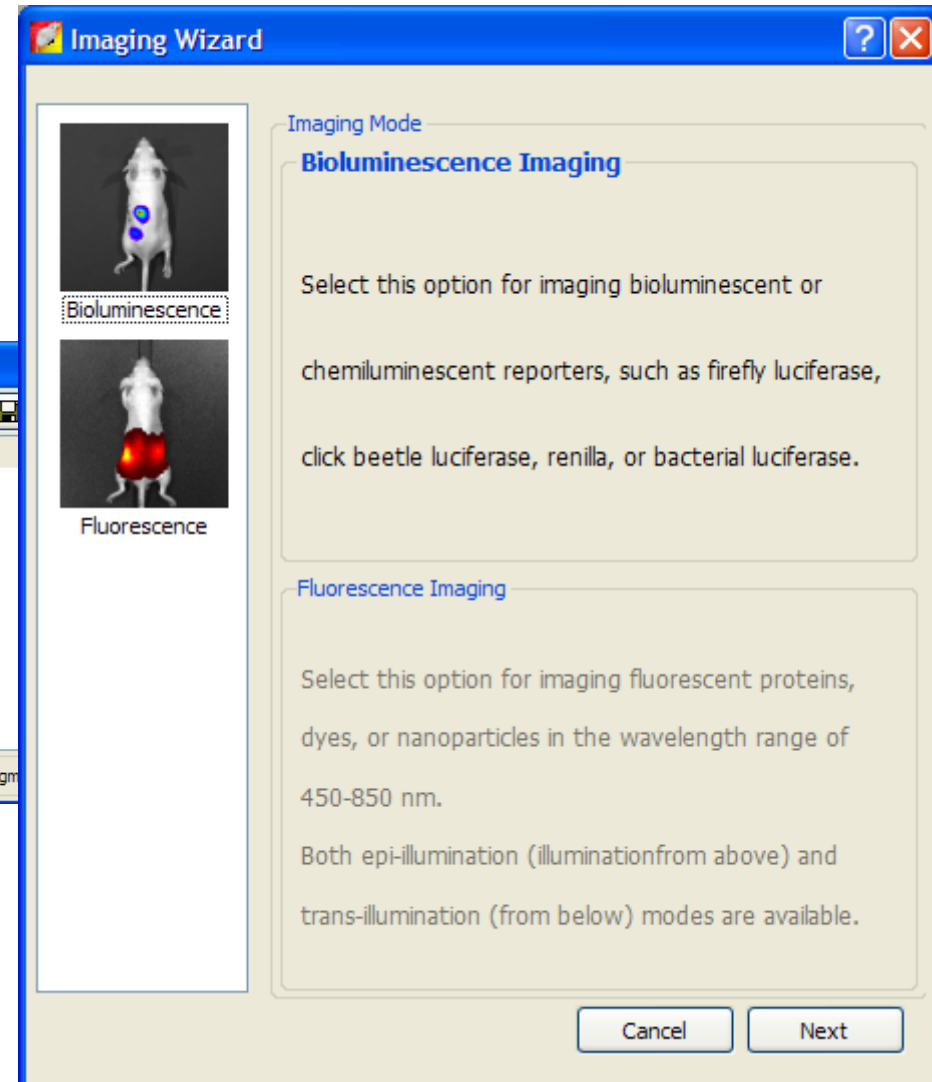
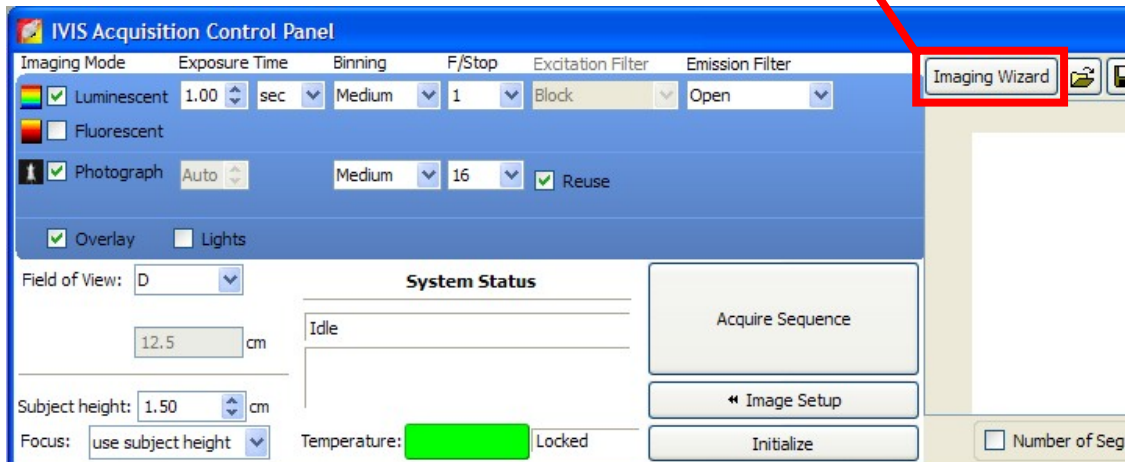
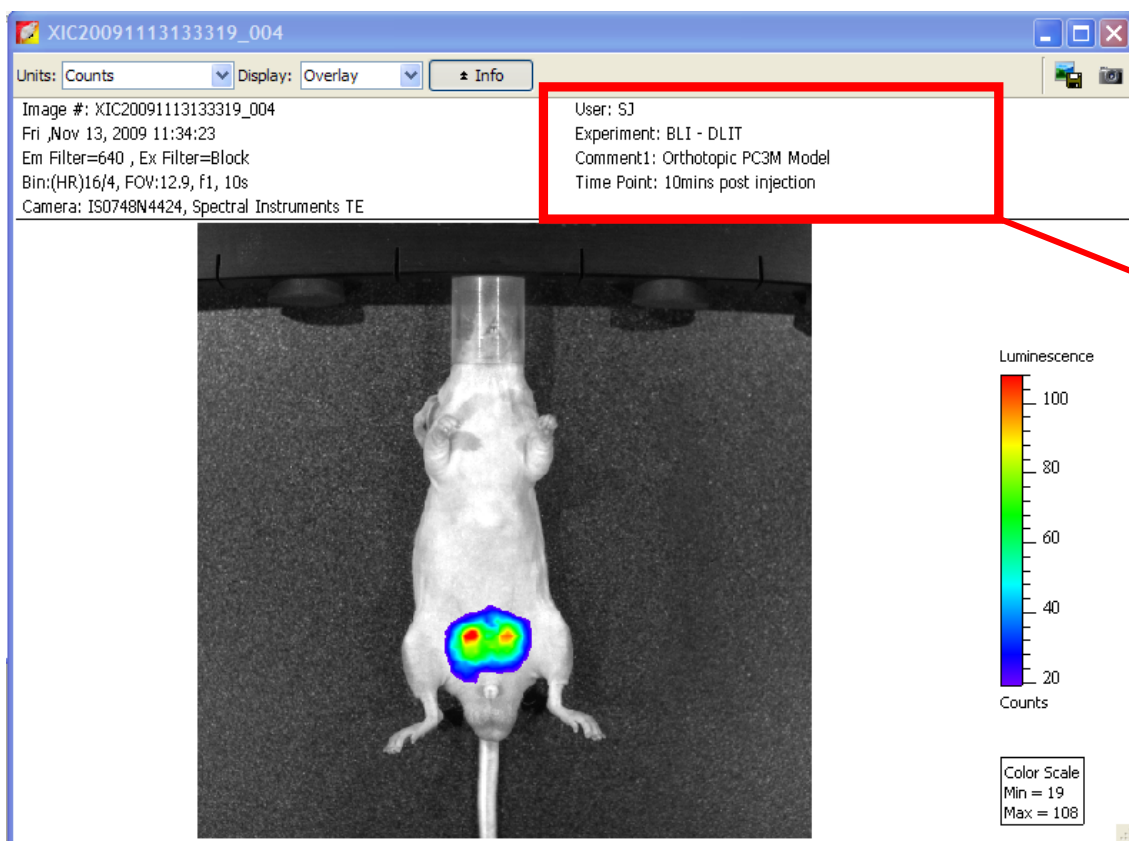


Image Labeling

- Good labeling practices are necessary for effective data browsing



Edit Image Labels
 UserID: XIC Living Image Universal
 Saved Labels: LABELS_1
 Check any 5 fields for display.

<input checked="" type="checkbox"/> User	SJ
<input type="checkbox"/> Group	
<input checked="" type="checkbox"/> Experiment	BLI - DLIT
<input checked="" type="checkbox"/> Comment1	Orthotopic PC3M Model
<input type="checkbox"/> Comment2	
<input checked="" type="checkbox"/> Time Point	10mins post injection
<input type="checkbox"/> Animal Number	
<input type="checkbox"/> Animal Strain	
<input type="checkbox"/> Animal Model	

Image Cataloging & Browsing Tools

Living Image® Browser

CK20090330114556_SEQ

Click Number	EX Fil	EM Fil	Illumination Modk	Use	User	Group	Experiment	Cor
CK20080731152638_SEQ					CK	XF750 pillows made 7/31/08	1.9e13/uL, 2:1 dilution, 2uL each	XF7
CK20080731155429_SEQ					CK	745/800 XF750 pillow in rod made 7/31/08	9.5e12/uL, 2uL each	XF7
CK20090330114556_SEQ					CK		PC3M-tdT Prostate Ortho Avastin-750 injection	Mou
CK20090330120010_SEQ					CK		PC3M-tdT Prostate Ortho Avastin-750 injection	Mou
TLT20050624145507_SEQ					TLT			
XIC20091113091104_SEQ					XIC		Tomato - pC3m	

Hide Browse View

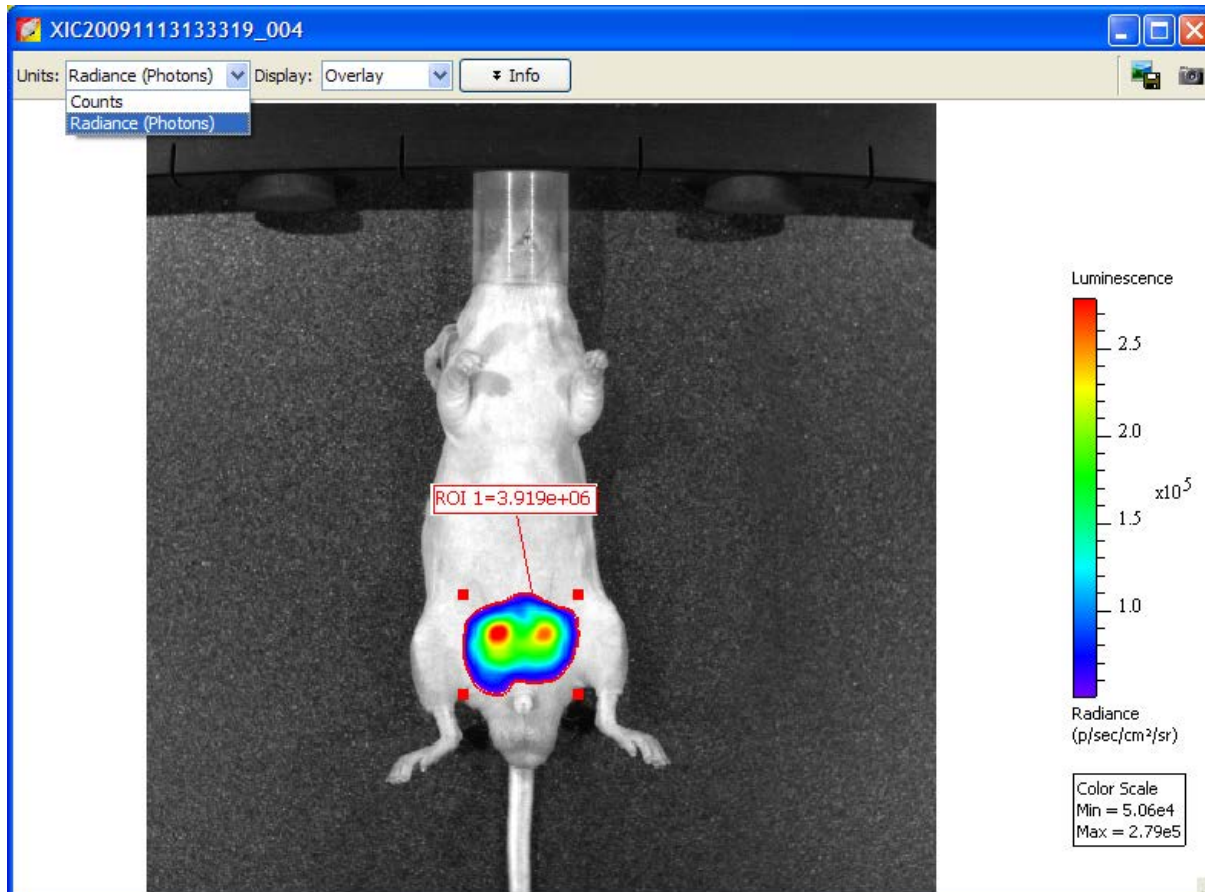
Close Preview Label Set: All Add to List Browse.. View: Default Configure

Location: C:/Documents and Settings/joness/Desktop/Xenogen/Training material/LI4 images/DLIT + FLIT/Avastin-AF-750 on PC3M-tdTomato/CK20090330114556_SEQ/SequenceInfo

← **User defined information** →

Quantification

- Tool palette for adjusting scale/opacity etc.
- Region of interest (ROI) tools to measure surface intensities



Regions of Interest Tools

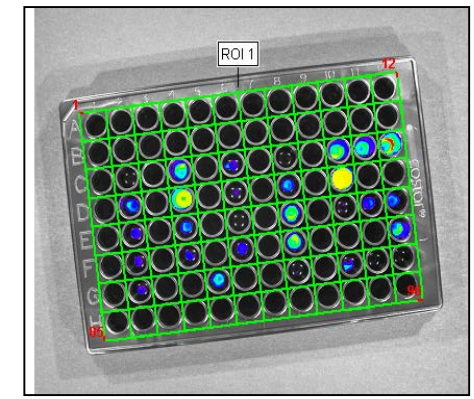
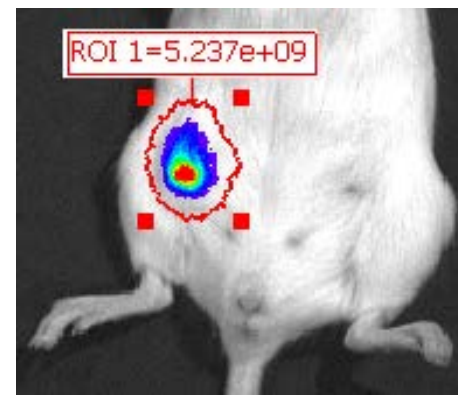
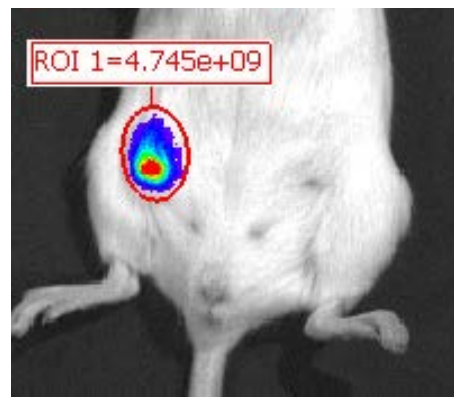
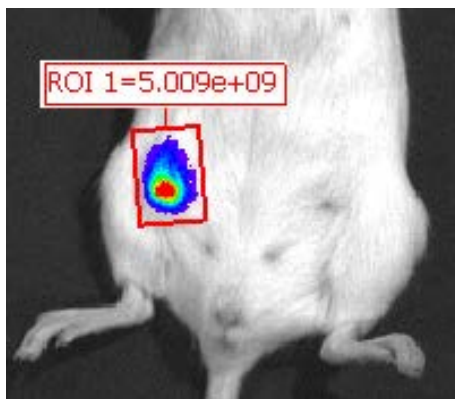
ROI shapes available:

- Square
- Circle
- Contour
- Grid

ROI's can be created:

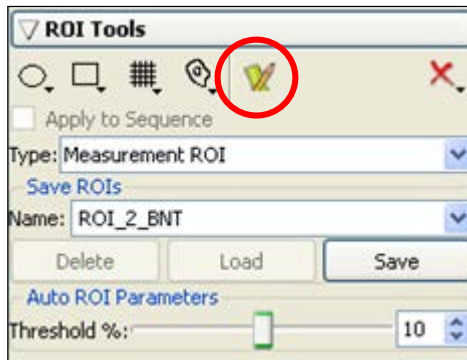
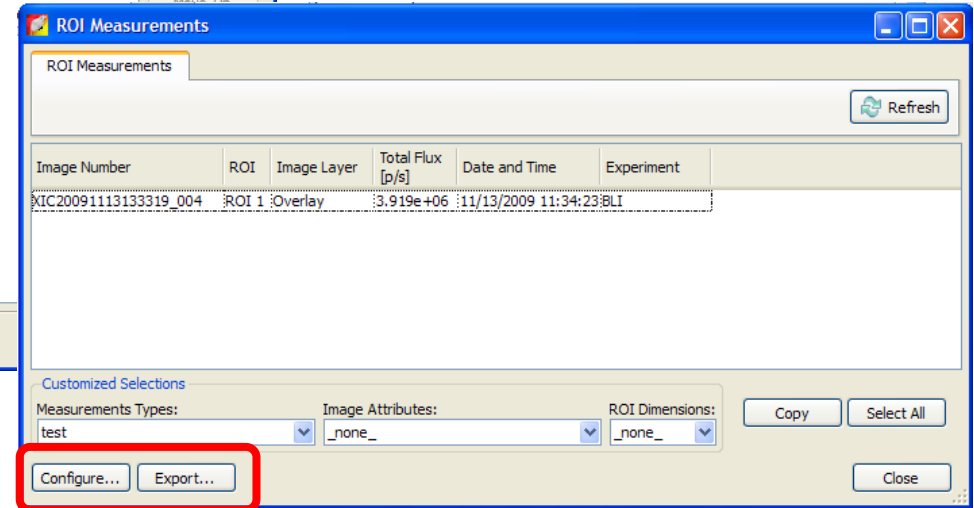
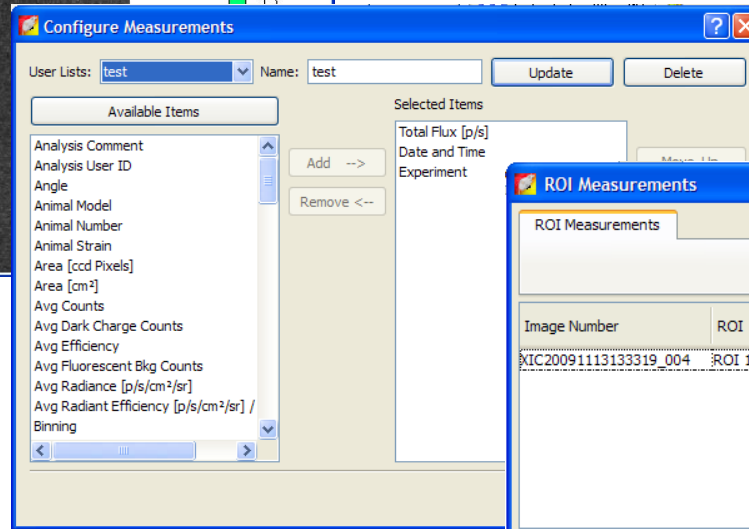
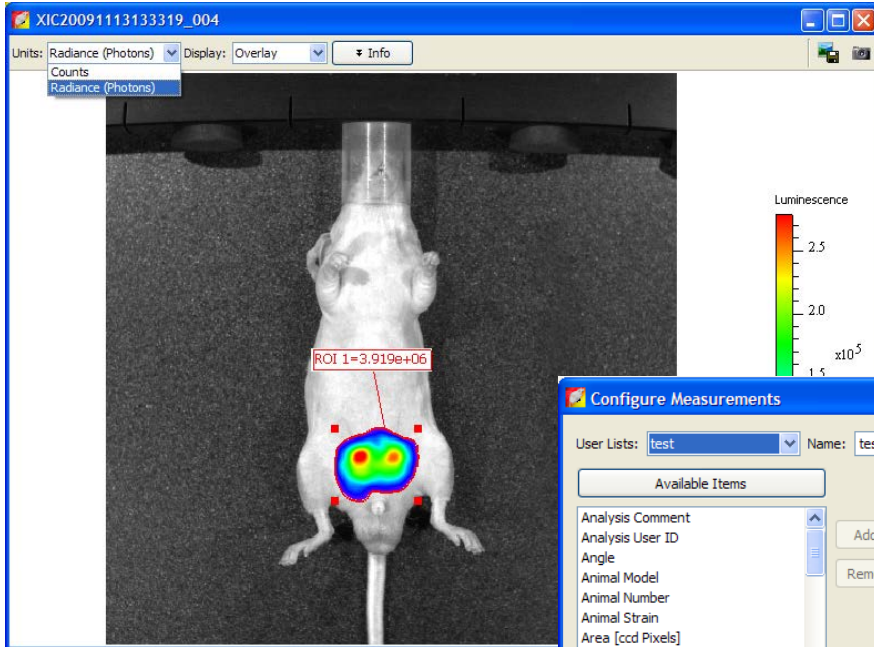
- Manually
- Automatically
- Free Draw

Important to be consistent with ROI selections



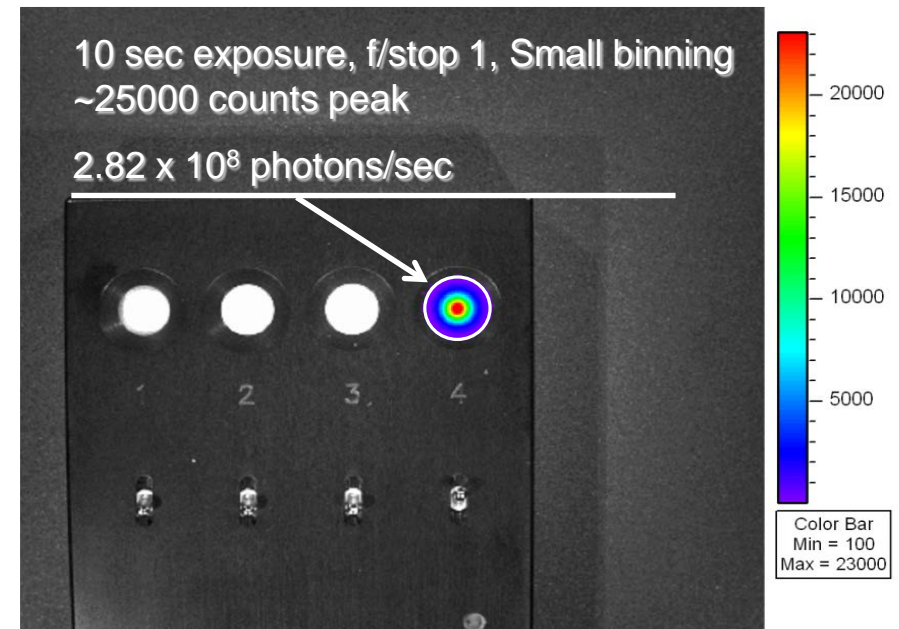
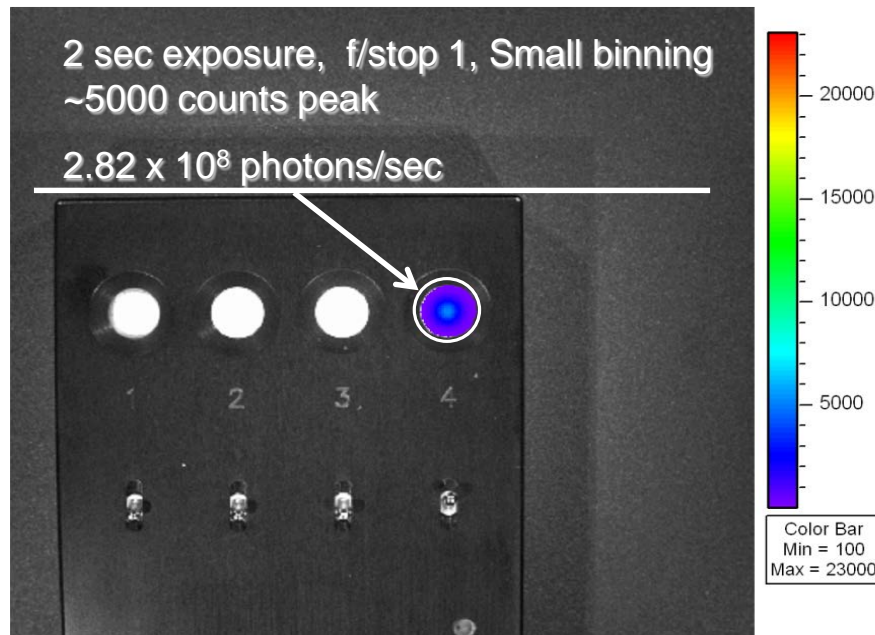
Measurement Table

- Measurement table displays information about each ROI
- Table is user configurable and can be exported to a spreadsheet



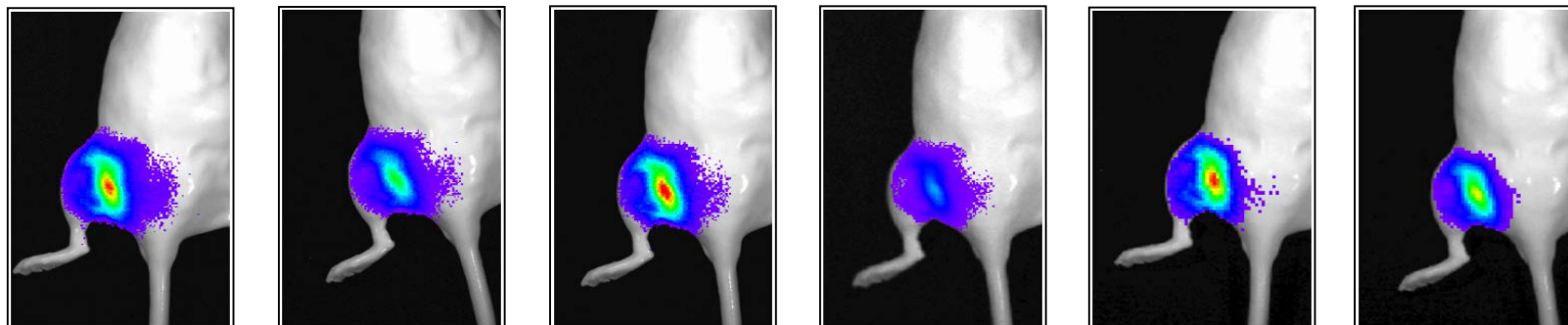
Calibrated Physical Units

- Living Image[®] automatically compensates for device settings: Exposure time, f/stop, Binning, and Field of View.
- Calibrated units are Photons per Second, representing the flux radiating omni-directionally from a user defined region.



Calibrated Physical Units vs Raw Signal - Example

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

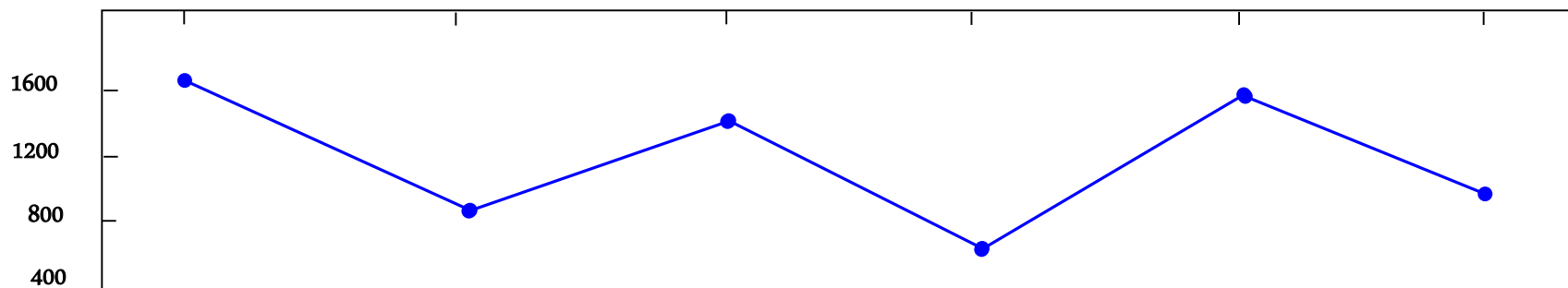
3

4

5

6

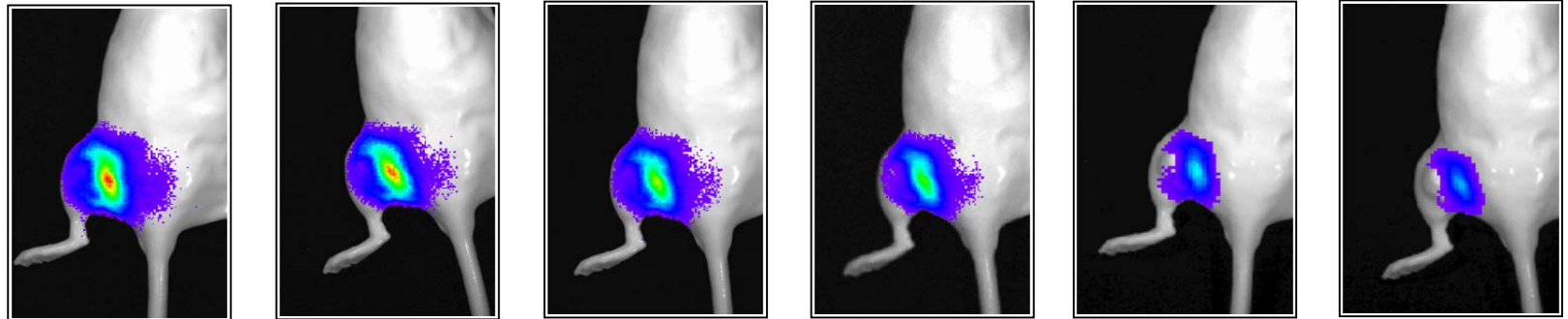
Peak Counts



Software - Analysis

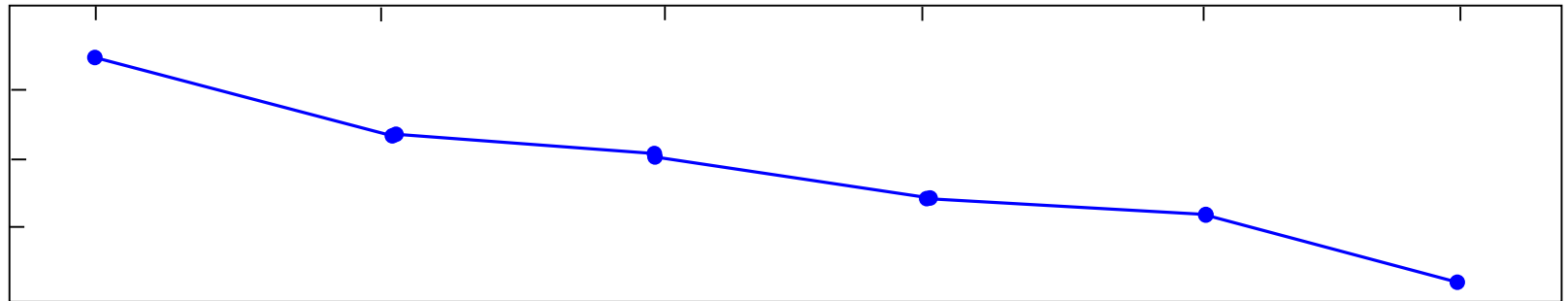
Calibrated Physical Units vs Raw Signal- Example

Calibrated Signal
(Photons per second)

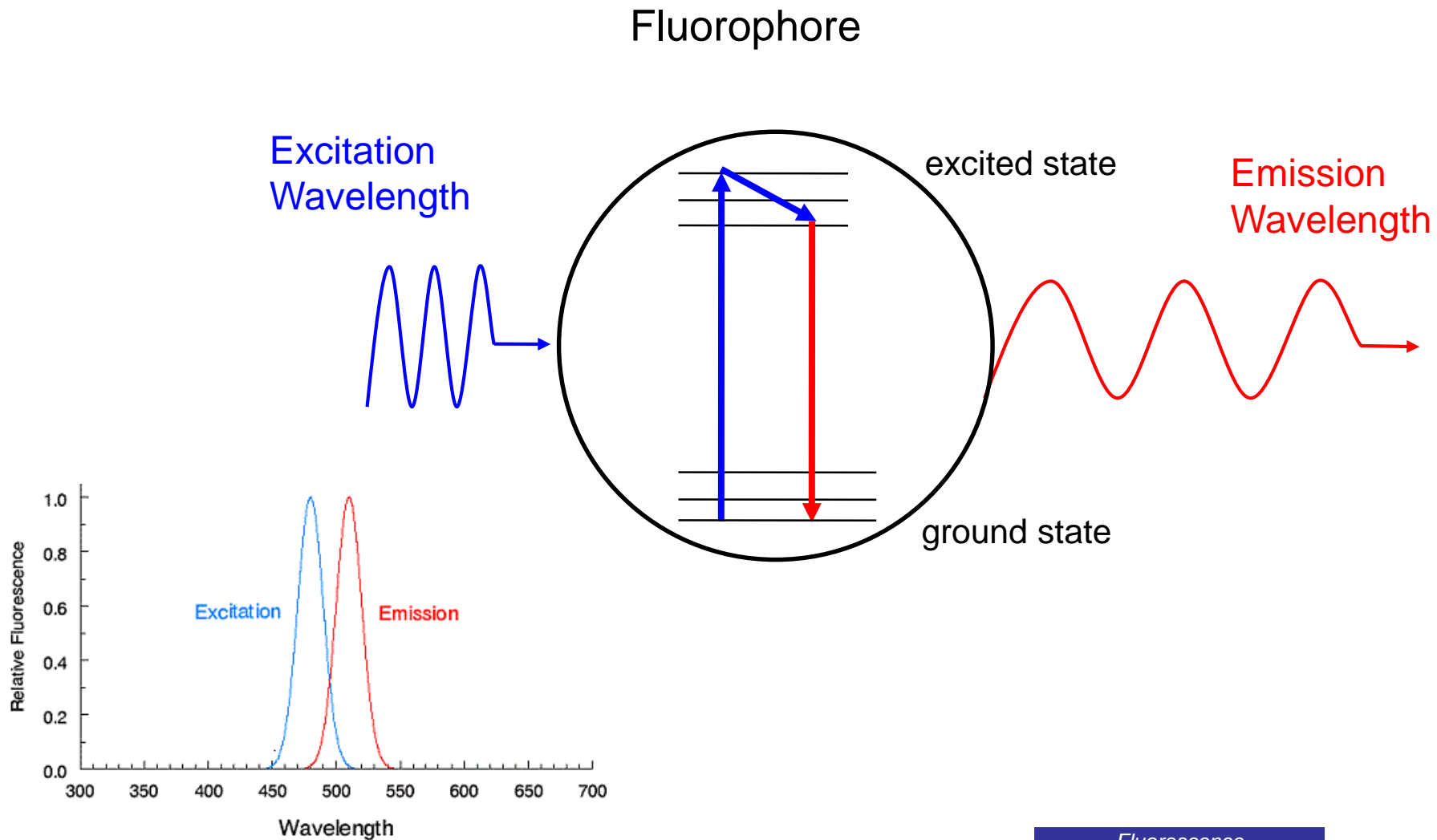


Exp time:	30 sec	30 sec	60 sec	60 sec	60 sec	60 sec
Binning:	small	small	small	small	medium	medium
Day:	1	2	3	4	5	6

Radiance: Photons
per second



Fluorescence

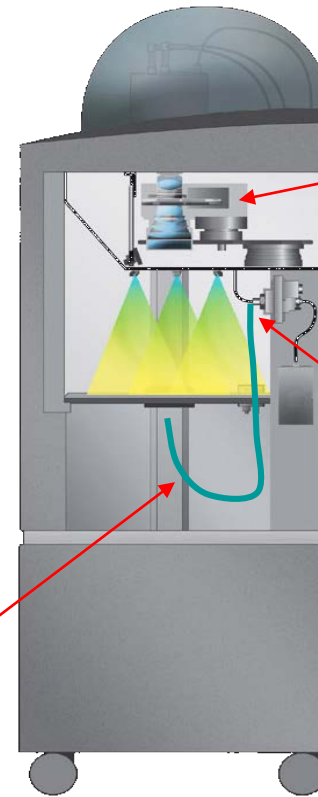


Fluorescence

IVIS[®] Fluorescence Imaging

- 18 Emission filters (computer controlled)
- 10 Excitation filters (computer controlled)
- 150 Watt Tungsten/Halogen lamp (computer controlled intensity)
- Low Auto Fluorescence optics and fibers

Trans-illumination
Light source



Emission
filter wheel

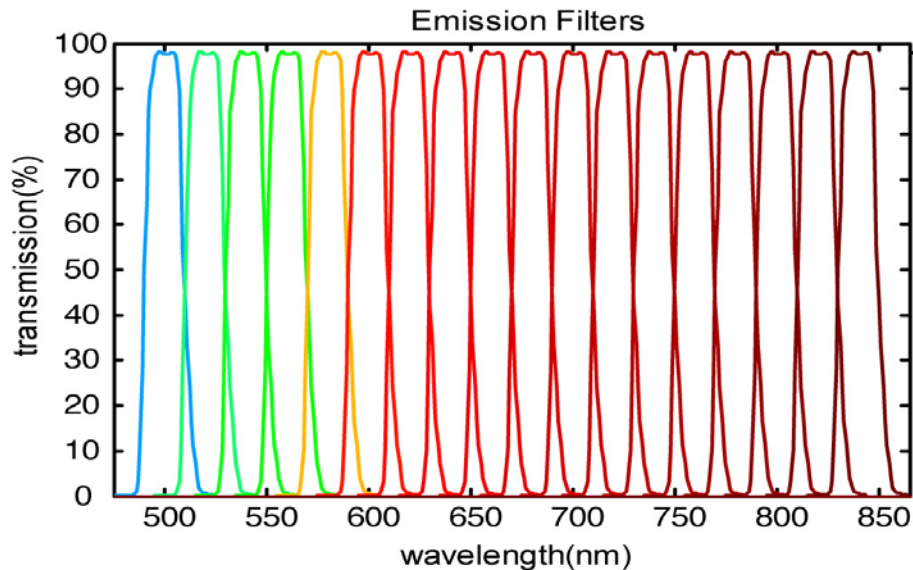
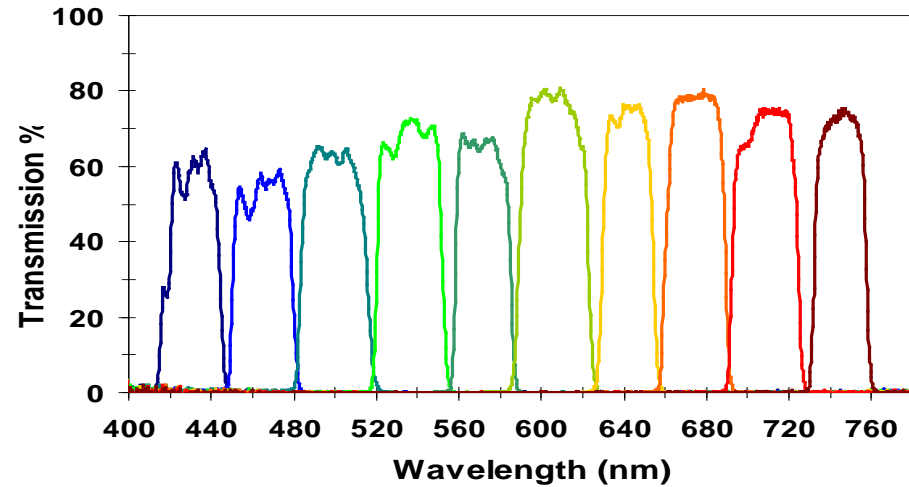
Excitation
filter wheel

Optical
switch

Fluorescence

Excitation and Emission Filters

10 Excitation filters –
35 nm bandwidth



18 Emission filters –
20 nm bandwidth

Fluorescence

Fluorescence Acquisition

Select Fluorescent
Imaging Mode

Trans-illumination

Lamp level
High / Low

Select filters

IVIS Acquisition Control Panel

Imaging Mode	Exposure Time	Binning	F/Stop	Excitation Filter	Emission Filter
<input type="checkbox"/> Luminescent	1.00 sec	Medium	2	535	Open
<input checked="" type="checkbox"/> Fluorescent	<input type="checkbox"/> Transillumination			Lamp Level: H	Open
<input checked="" type="checkbox"/> Photograph	Auto	Medium	8		Empty
<input type="checkbox"/> Structure					500
<input checked="" type="checkbox"/> Overlay					520
<input type="checkbox"/> Lights					540
<input checked="" type="checkbox"/> Alignment Grid					560
					580
					600
					620
					640
					660

System Status
Idle

Service: 12.9 cm

Subject height: 1.50 cm

Focus: use subject height

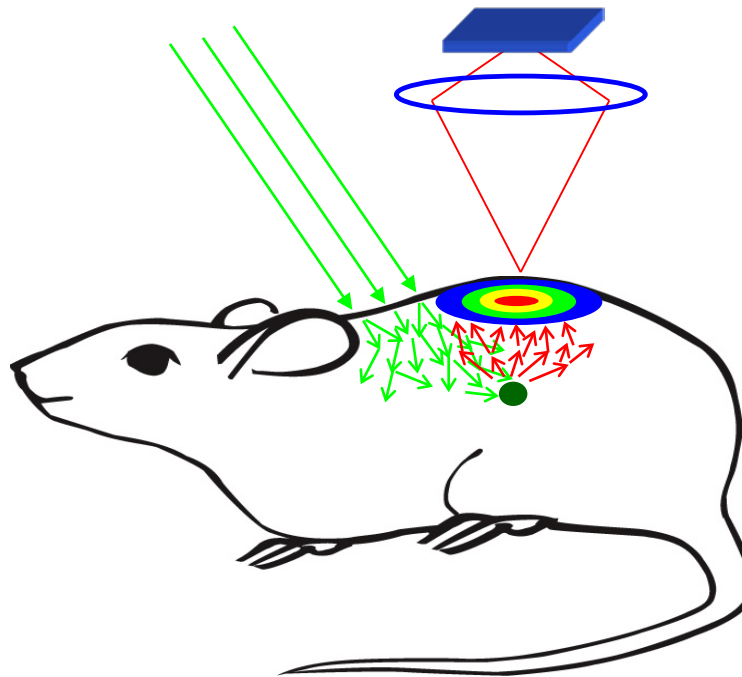
Temperature: [Redacted] Locked

Buttons: Sequence Setup, Initialize

Fluorescence

Fluorescent Calibrated Units: Radiant Efficiency

$$\text{Radiant Efficiency} = \frac{\text{Emission Light (photons/sec/cm}^2\text{/str)}}{\text{Excitation Light } (\mu\text{W/cm}^2)}$$



Fluorescence

Fluorescent Calibrated Units: Radiant Efficiency

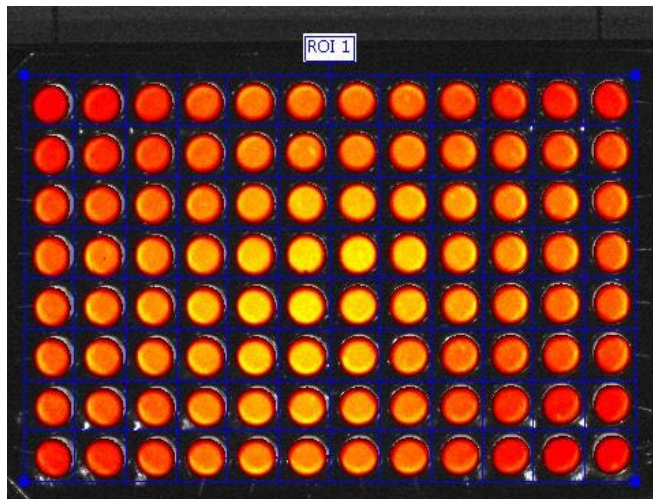
Excitation
Light Pattern



*Units of 'Radiant Efficiency'
compensates for non-uniform excitation
light pattern*

GFP Well Plate Uncorrected

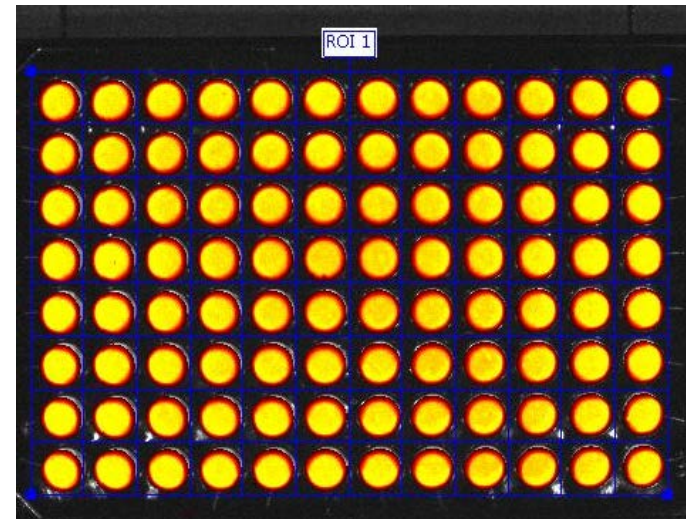
Counts



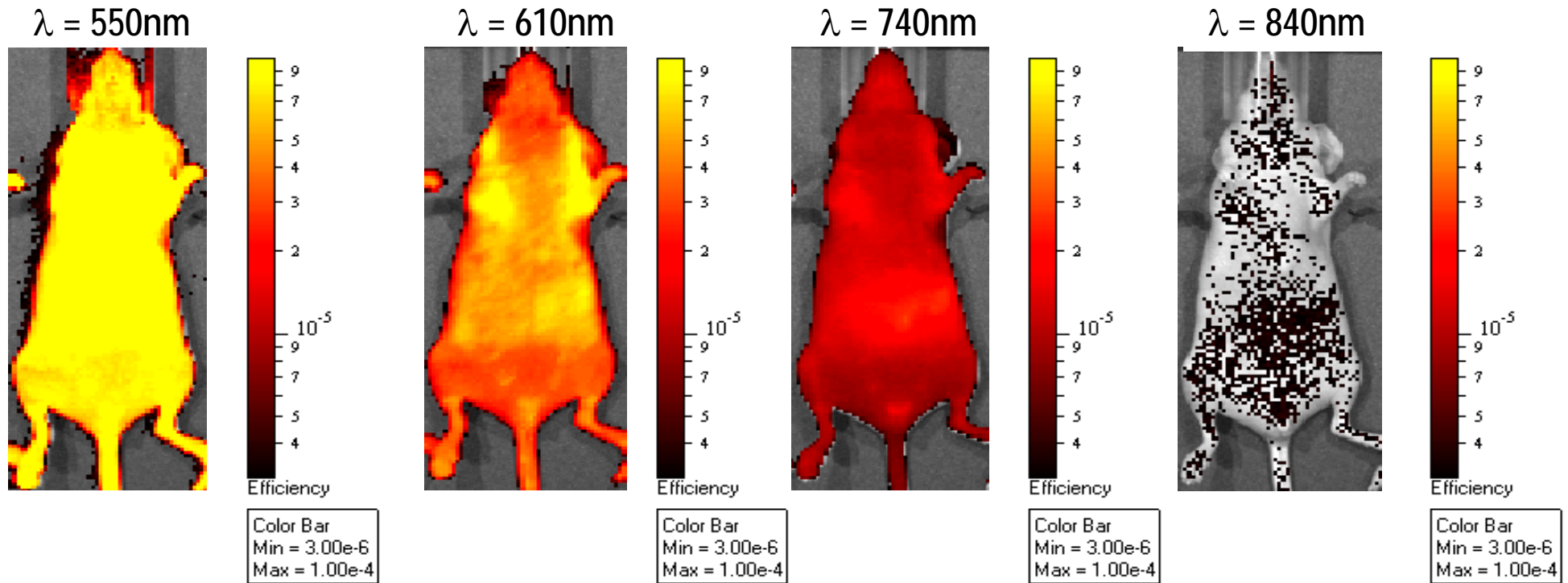
vs.

GFP Well Plate Corrected

Radiant Efficiency



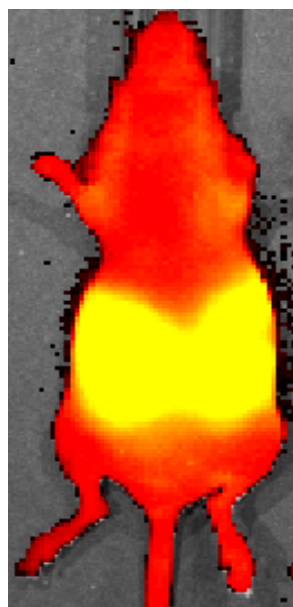
Auto-fluorescence of Control Mice



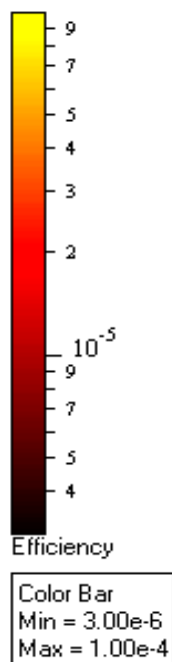
Fluorescence

Animal Diet Auto-fluorescence in Control Mice

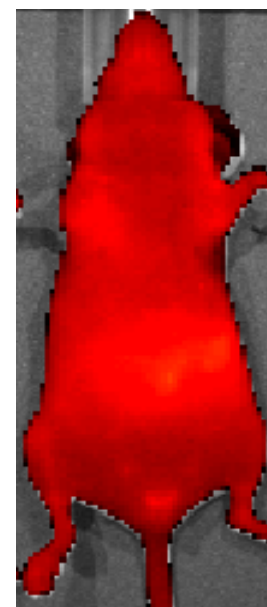
Regular Rodent Food



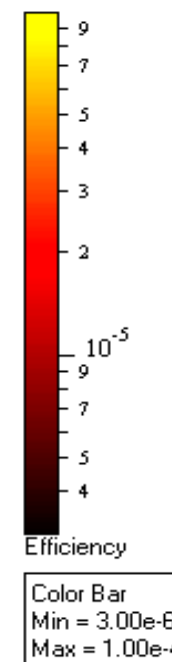
$\lambda = 740\text{nm}$



Alfalfa Free Rodent Food

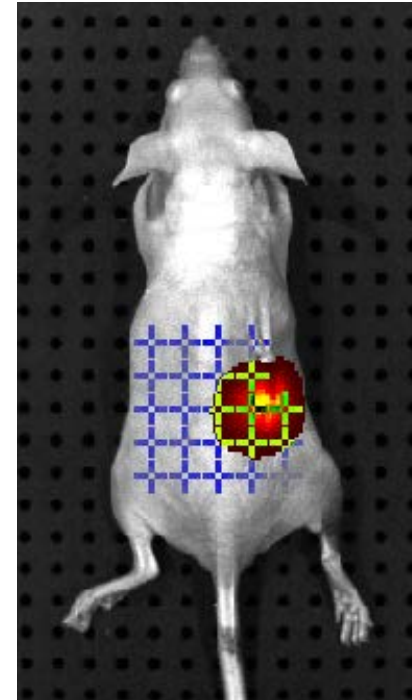


$\lambda = 740\text{nm}$



Fluorescence

Transmission Fluorescence

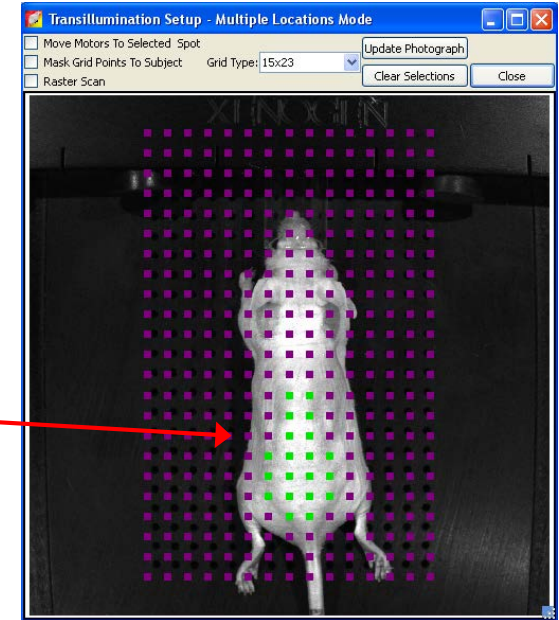


- Optimized for imaging deep signals, improves signal/noise

Trans-illumination Sequence

Highlight multiple locations of interest (or entire subject) and add to the image sequence

Select trans-illumination points



IVIS Acquisition Control Panel

Imaging Mode: Luminescent Fluorescent Photograph Structure

Exposure Time: Auto sec Binning: Medium F/Stop: 2 Excitation Filter: 710 Emission Filter: 800

Transillumination Normalized Reuse

Lamp Level: High

Field of View: C

System Status: Idle

Service: 12.9 cm

Subject height: 1.50 cm

Focus: use subject height Temperature: Locked

Acquire Sequence

Image Setup

Initialize

Imaging Wizard Display Photographic Settings Subject: None Probes

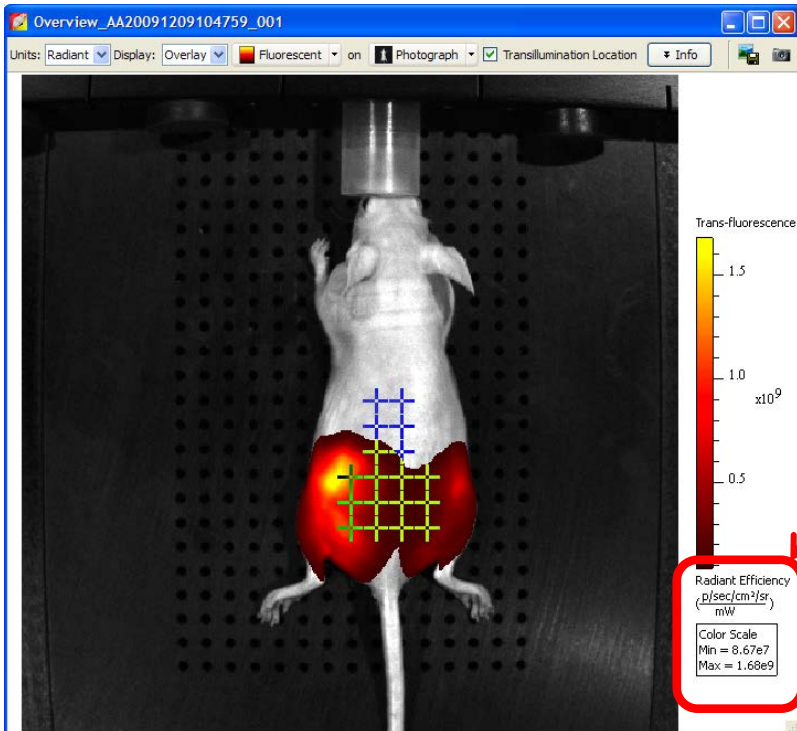
Mode	Exposure	Binning	FStop	Excitation	Emission	Lamp Level	Structure	FOV	Height	Transillumination	De
1	Auto	Medium	2	710	800	High	No	C	1.50	15x23:19, 6	
2	Auto	Medium	2	710	800	High	No	C	1.50	15x23:19, 7	
3	Auto	Medium	2	710	800	High	No	C	1.50	15x23:19, 8	
4	Auto	Medium	2	710	800	High	No	C	1.50	15x23:19, 9	
5	Auto	Medium	2	710	800	High	No	C	1.50	15x23:19, 10	
6	Auto	Medium	2	710	800	High	No	C	1.50	15x23:18, 6	
7	Auto	Medium	2	710	800	High	No	C	1.50	15x23:18, 7	
8	Auto	Medium	2	710	800	High	No	C	1.50	15x23:18, 8	

Number of Segments: 1 Delay: 0.0 min Apply to All Remove Update Insert Add

Trans-illumination units

Imaging units are defined as **Radiant Efficiency**;

Emission Radiance ÷ Excitation light power



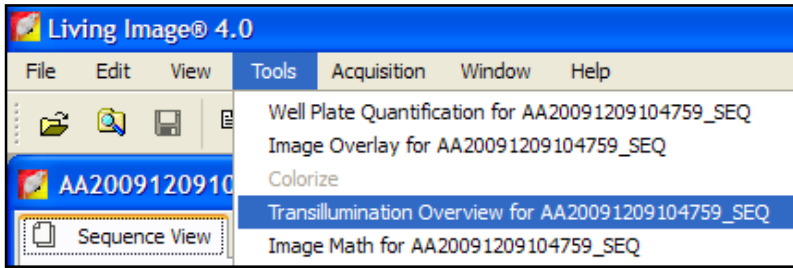
Radiant Efficiency
($\frac{\text{p/sec/cm}^2/\text{sr}}{\text{mW}}$)

Color Scale
Min = 6.27e6
Max = 3.98e7

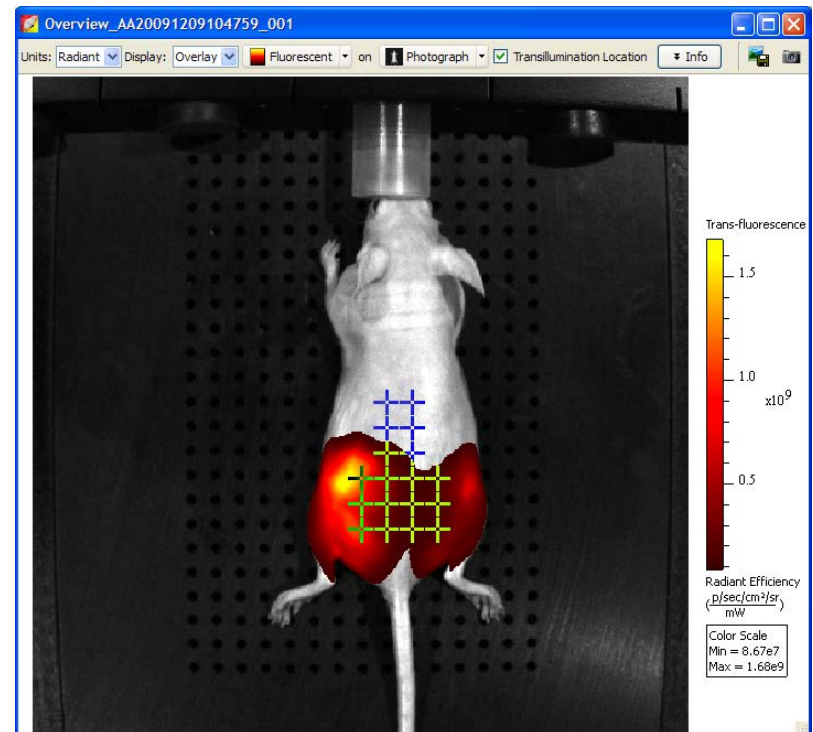
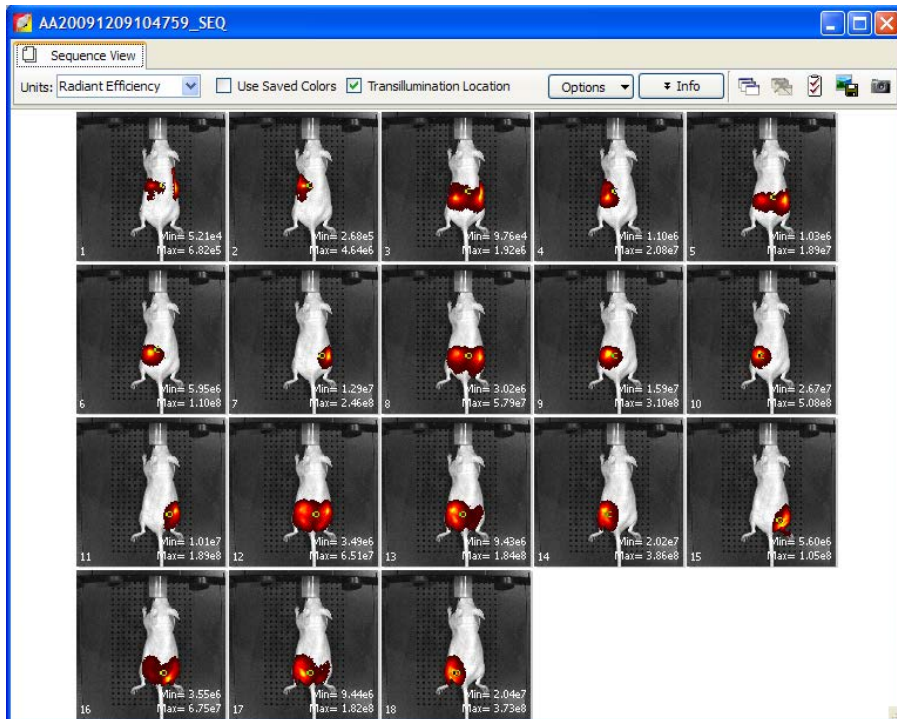
Note:
Quantification not comparable
to Epi-Fluorescence images;

Emission Radiance ÷ Excitation power density (per
area)

Trans-illumination Sequence Overview



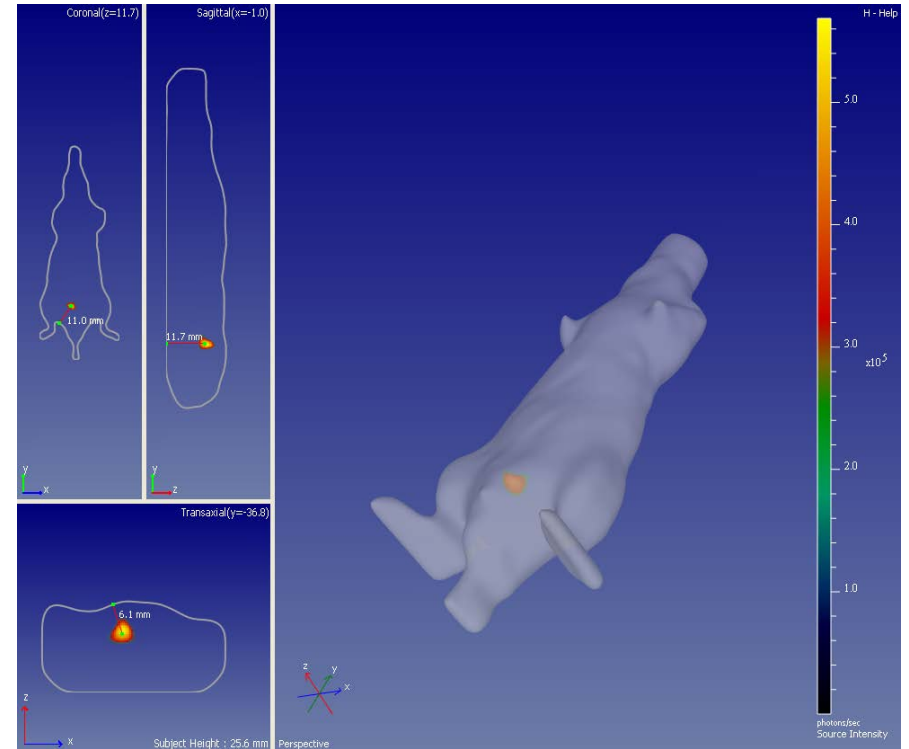
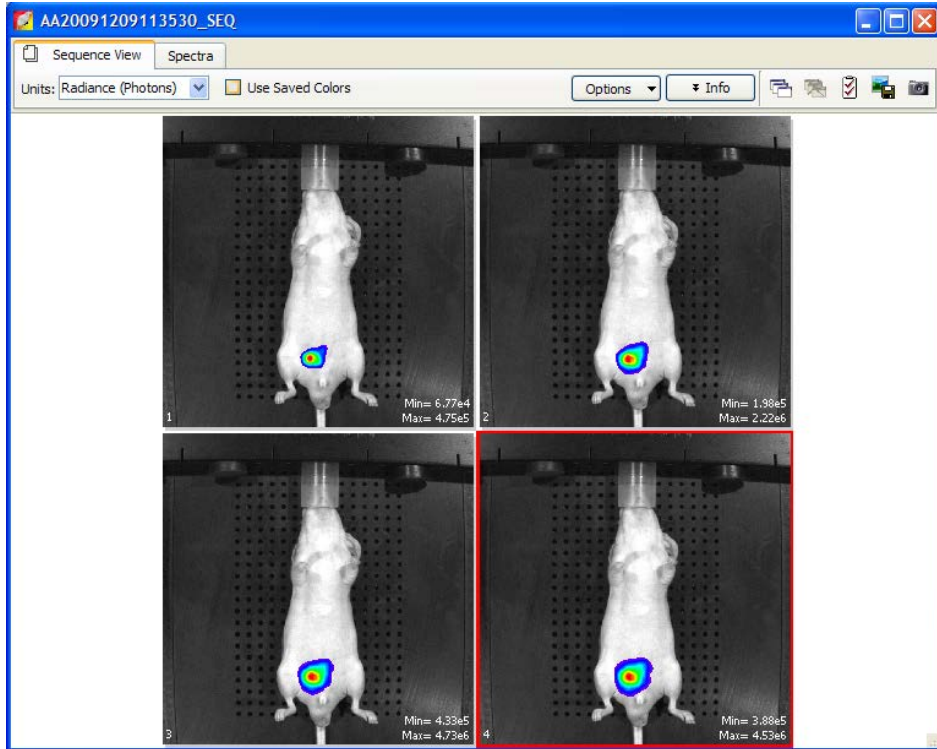
Combined image overview of multiple trans-illumination images



Advanced topics (preview)

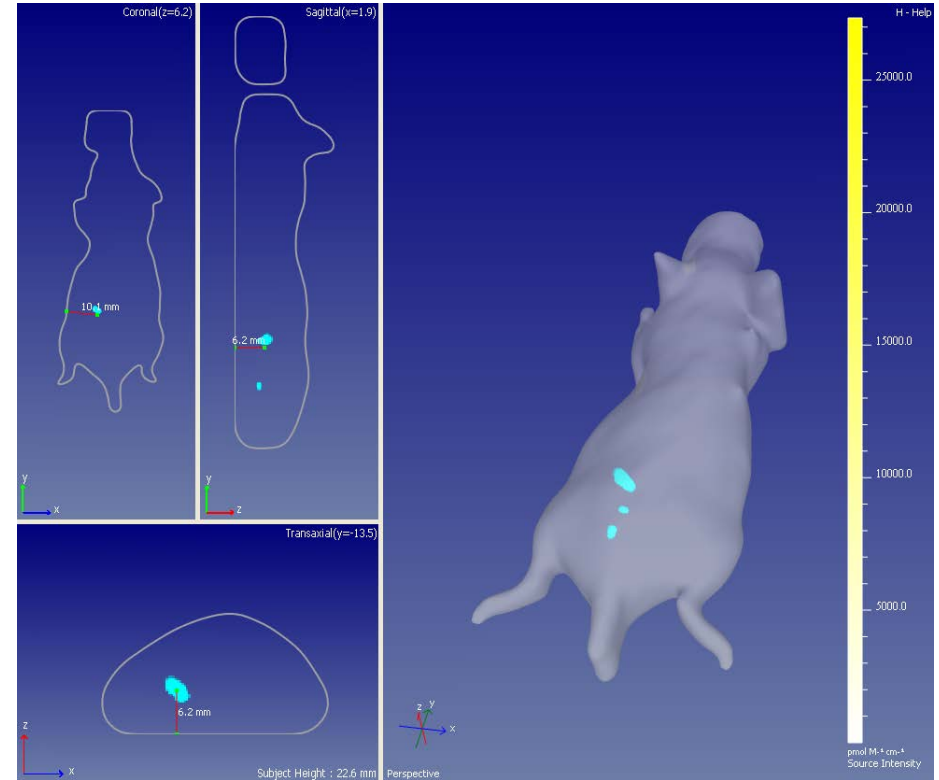
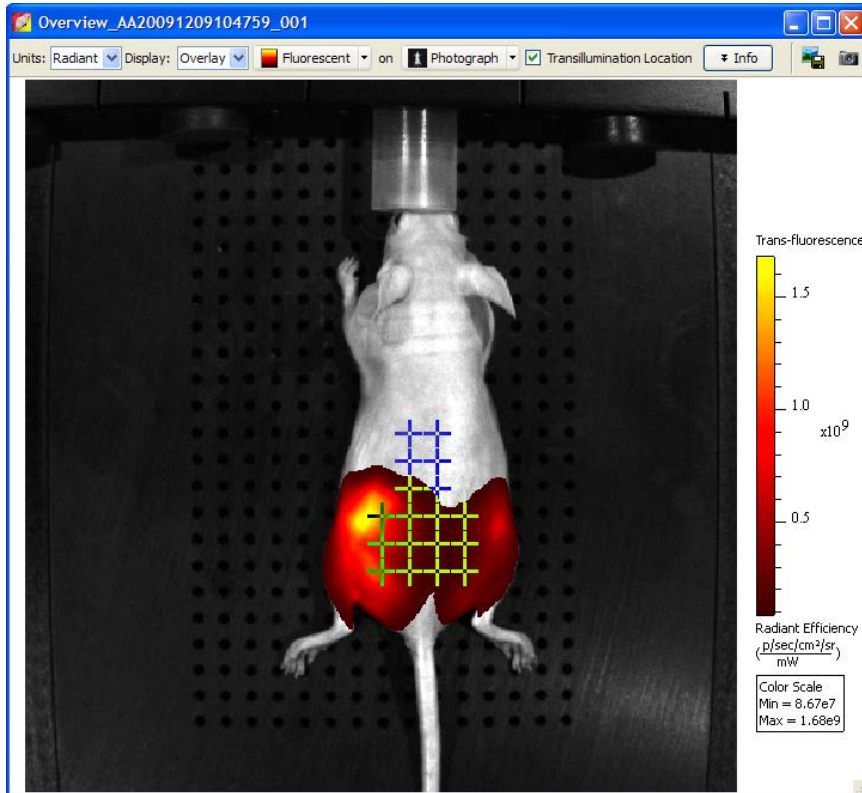
- 3D Tomography (Luminescence and Fluorescence)
- Spectral Un-mixing

3D Tomography (Luminescence)



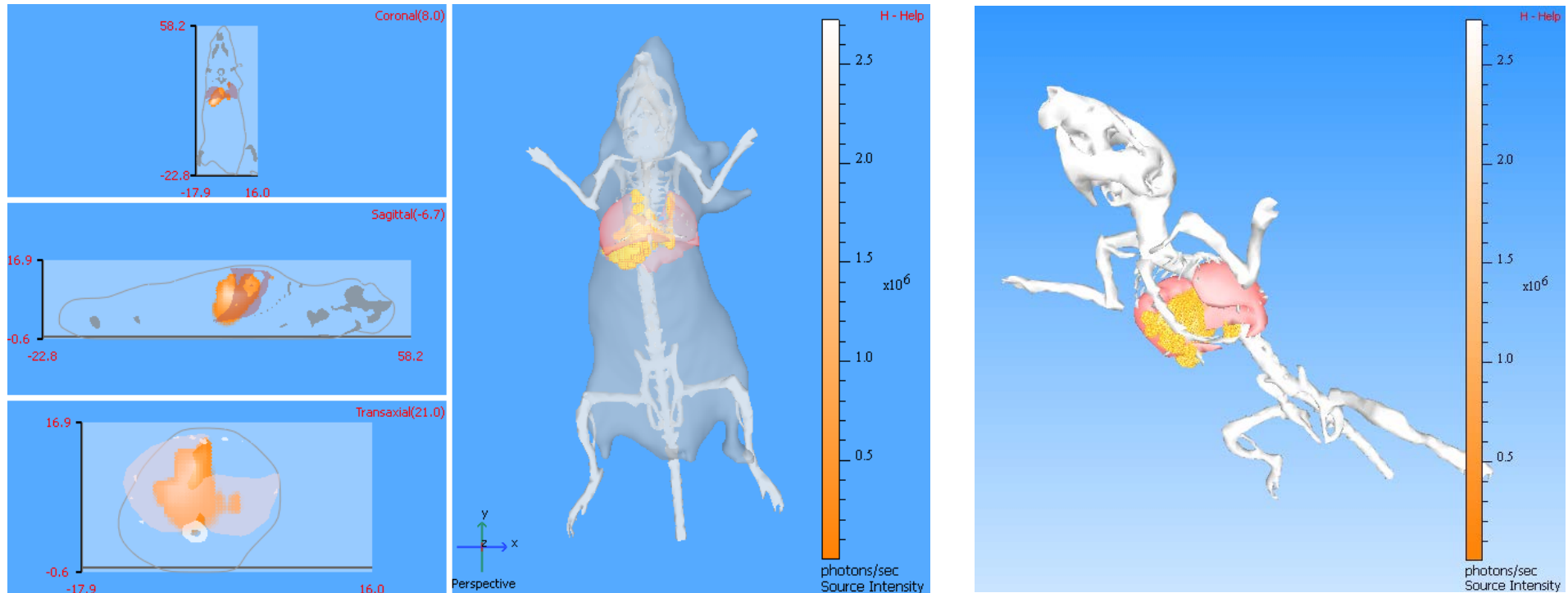
Spectral scans with Emission filters allows for absolute quantification and source localization

3D Tomography (Fluorescence)



Spatial scans with trans-illumination light source allows
for absolute quantification and source localization

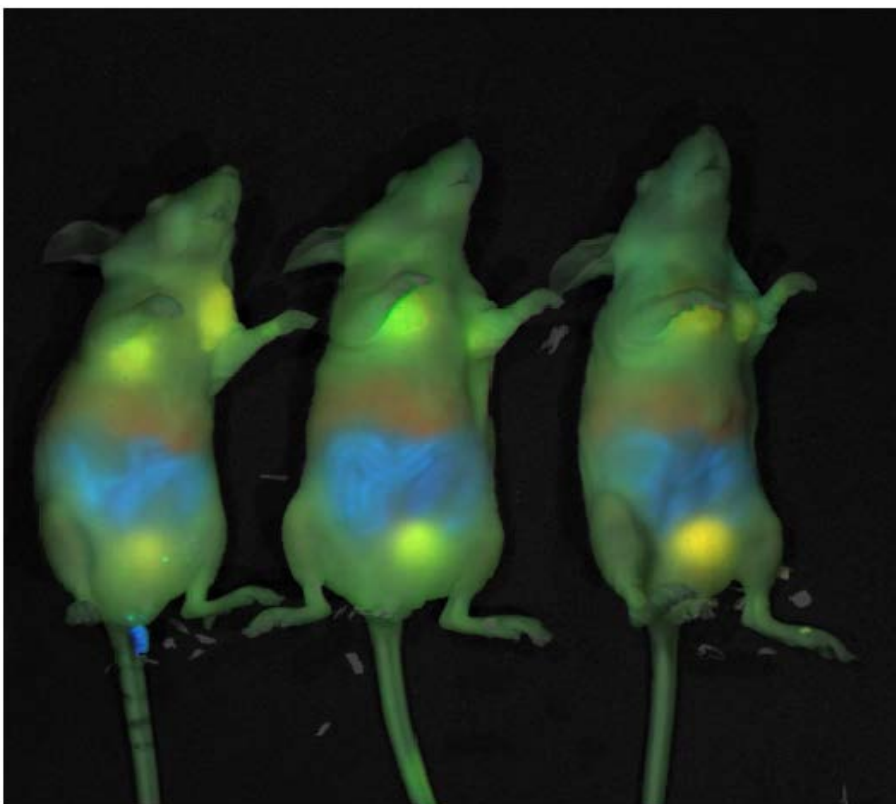
3D Tomography with co-registration - μ CT and organ atlas database



Ability to add and co-register images from a library of μ CT / atlas data plus import/export DICOM formatted files

Spectral Un-mixing

- Ability to calculate contributions from different fluorescent components (inc. auto-Fluorescence)



Composite image of 4T1 murine mammary tumor cells implanted in mammary fat pads:

- ProSense 680 (Yellow) activated by cathepsins in tumor cells and accumulates in bladder
- MMPsense750 (Red) activated by metalloproteinases in tumor cells and liver accumulates in bladder
- Auto-fluorescence from chlorophyll in food (blue) and animal tissue background (green)

Summary

Imaging principles

- Light is scattered and absorbed by tissue - dependant on wavelength and depth
- **Calibrated physical units compensate for device settings**

Hardware

- Custom designed for *in-vivo* bioluminescent & fluorescent imaging
- 28 filters make IVIS Spectrum ideal for imaging multiple probes
- Settings are analogous to photography

Software

- Living Image[®] used for acquisition and analysis
- Images are acquired in a two step process
- **Sensitivity is controlled by Exposure time, f/stop and binning**

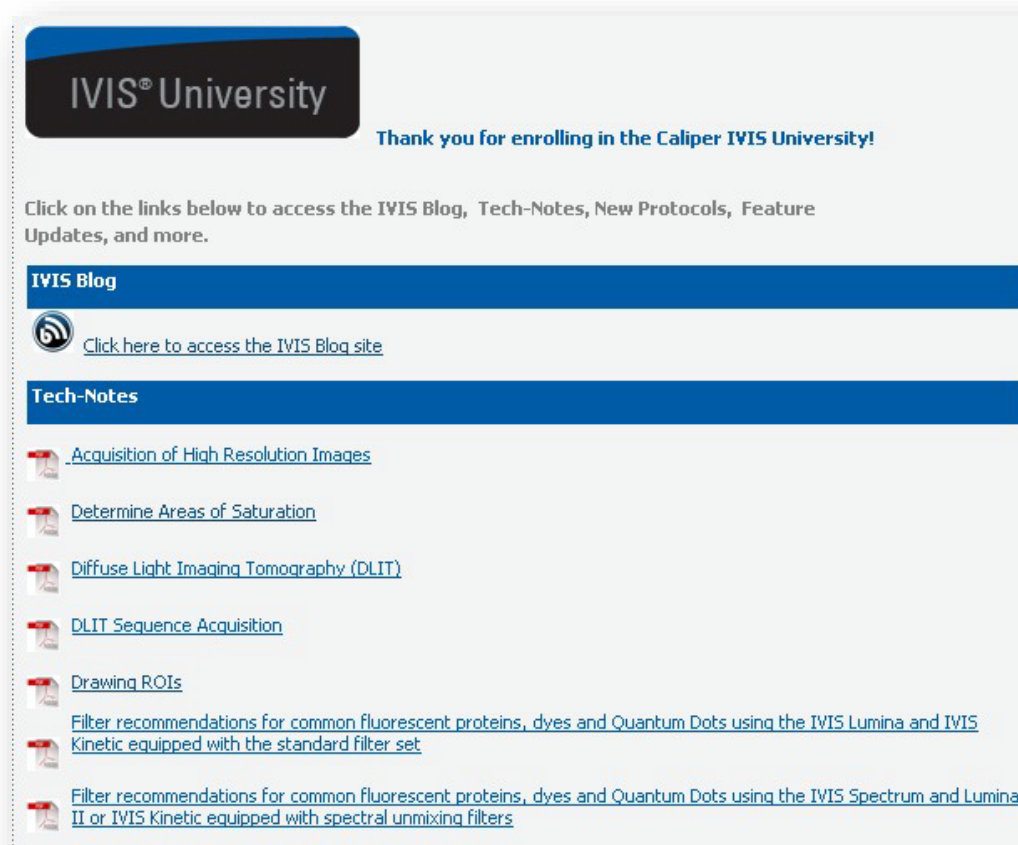
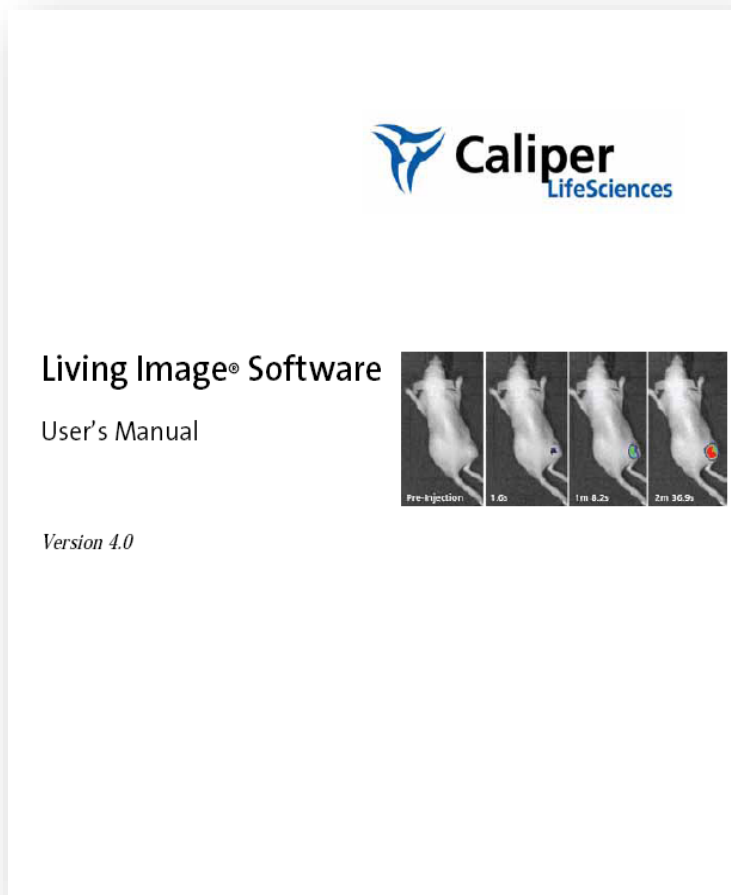
Fluorescence

- **Two modes of illumination: Reflection (epi) or Transmission**
- Tissue and Instrument Auto-fluorescence can be subtracted

For an In Depth Study

IVIS Software Manual

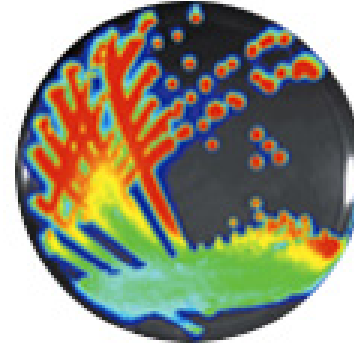
IVIS University Web page
www.caliperls.com/products/optical-imaging/ivis-university.php



Software



IVIS | Bioware and Reagents



- ✓ Bioware
- ✓ Bioware Ultra
- ✓ Bioware Ultra Red

IVIS | XenoLight



- ✓ NIR Fluorescent Reagents 680, 750, 770nm Protein Labeling Kits

- ✓ DiR

Suzen O'Coin
(508) 497-6489
suzen.ocoin@caliperls.com



- ✓ D-Luciferin Substrate



- ✓ Rediject D-Luciferin
- ✓ Rediject D-Luciferin Ultra