

## In-Vivo Imaging: IVIS Spectrum



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## What will be covered?

### Introduction

- Principles of Optical 'In Vivo' Imaging
- Key IVIS<sup>®</sup> Hardware components
- Overview of Living Image<sup>®</sup> 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

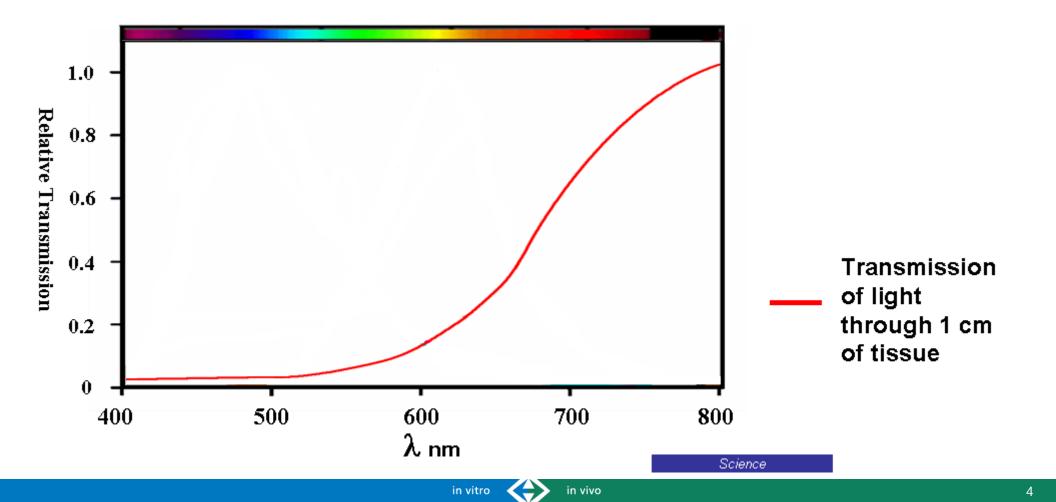
• Relatively simple instrumentation.

in vivo

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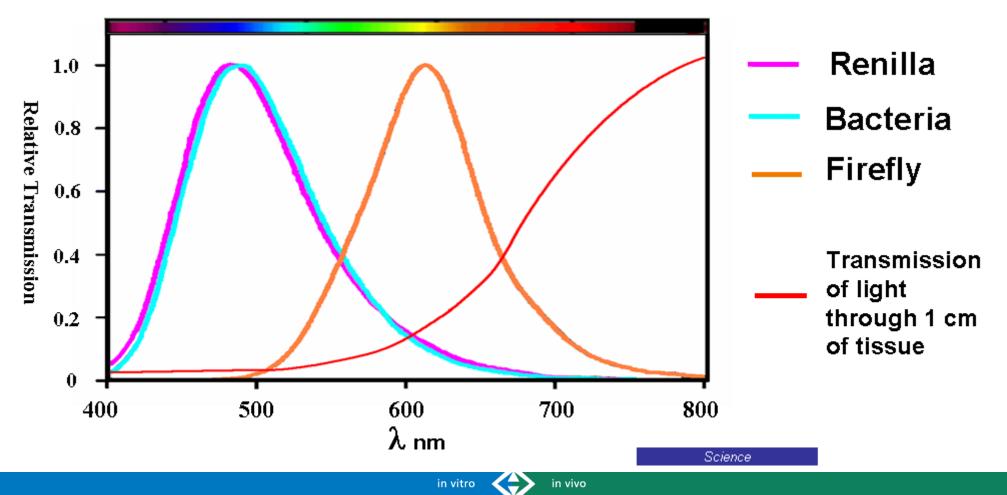
### Tissue is not Transparent - Light Absorbance Depends on Wavelength



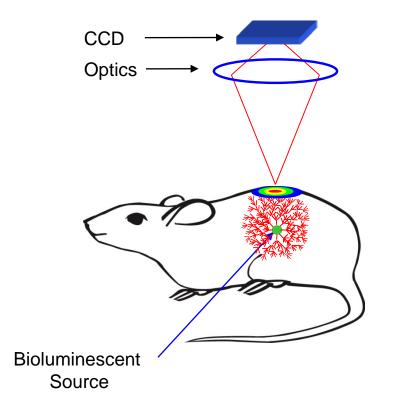
## Tissue is not Transparent - Light Absorbance Depends on Wavelength

Caliper

Luciferase Emission Spectra and Tissue Transmission



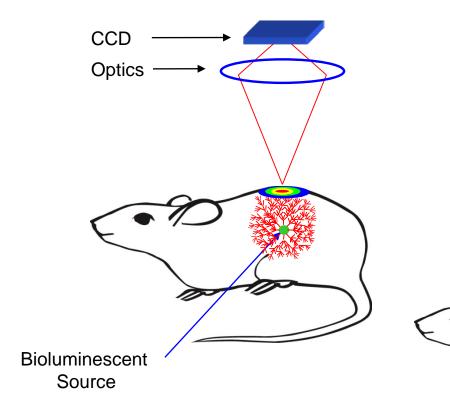




Caliper

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





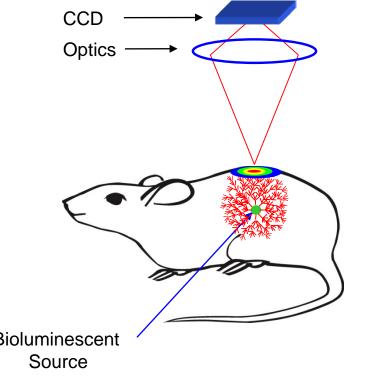
Caliper

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**Bioluminescent** 

Caliper

in vivo

in vitro

8



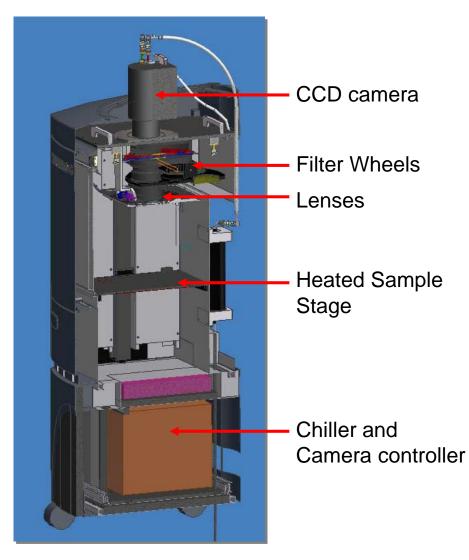
### **IVIS®** Spectrum Imaging System - Hardware

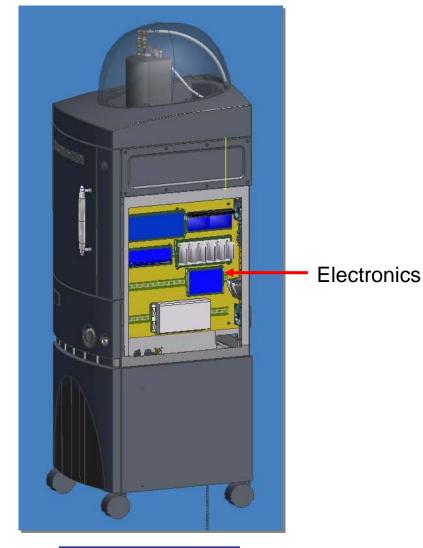


- Customized for *in vivo* imaging
- Highly sensitive camera with a large dynamic range



# **IVIS<sup>®</sup> Spectrum Imaging System – Hardware**



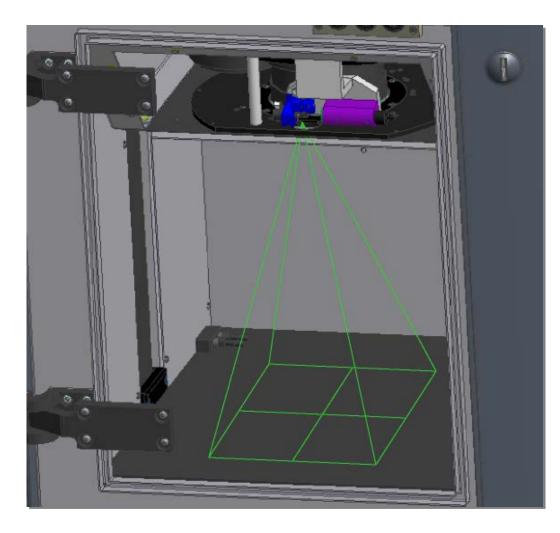


Hard<u>ware</u>

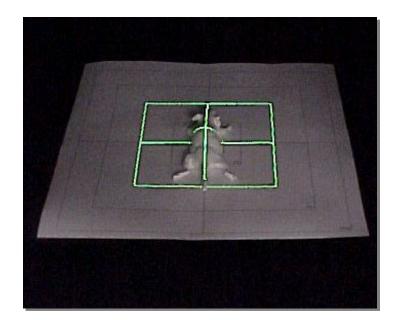




### **Alignment Light Projector**



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







## Living Image<sup>®</sup> Software

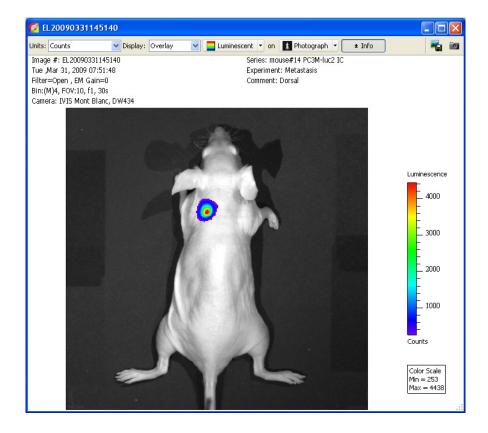
- Controls all settings in the IVIS<sup>®</sup> system (fully computer controlled)
- Provides advanced cataloging and browsing tools
- Provides analysis tools for quantification
- Instrument settings are analogous to photography
- Images are acquired in a two step process





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

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(2)	ue ,Mar 31, 2009 07:51:48 ilter=Open , EM Gain=0 in:(M)4, FOV:10, f1, 30s	Experiment: Metastasis	
		0	4000  3000





in vivo

Color Scale Min = 253 Max = 4438

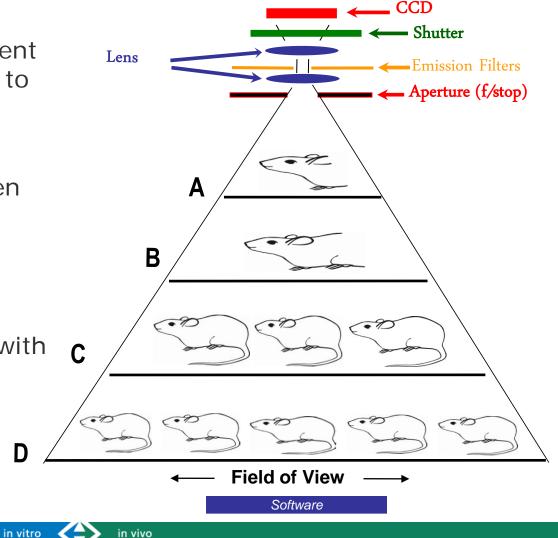
### Camera and Lens Settings are Analogous to Those Used in Standard Photography

D

 Field of View (FOV) is dependent on the distance from the lens to the sample

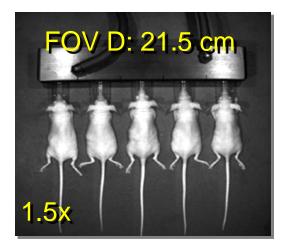
Caliper

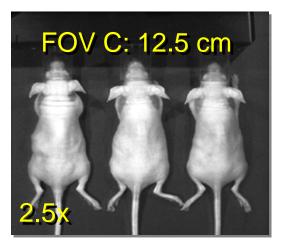
- Light collected is proportional to how long the shutter is open (exposure time)
- Aperture (f/stop) controls the amount of light collected
- Digital pixel binning possible with CCD - for further increase in sensitivity

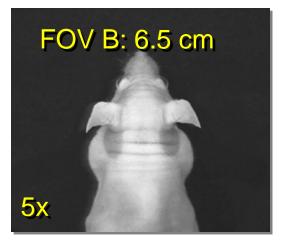


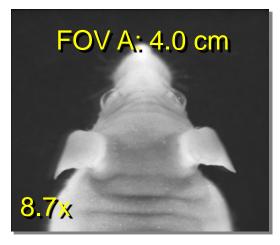


### Field-of-View (typical)













### Setting Sensitivity – Luminescent Signal Level

- The IVIS<sup>®</sup> CCD camera has a <u>raw</u> signal range of 0 to 65535 Analog to Digital Counts (2<sup>16</sup>).
- 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** 

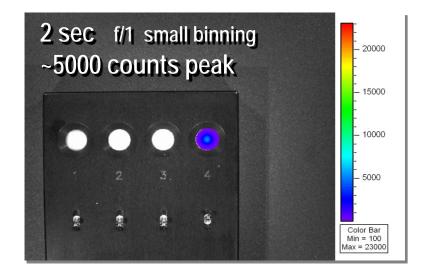
💋 IVIS Acqu	uisition (	Control	Pan	÷						
Imaging Mode	Exposure Time	Binning		F/Stop	Excitation Filter	E	Emission Filter			
📃 🗹 Luminescent	1.00 🤤 sec	🔽 Medium	¥	1 💌	Block	Y	Open	*		
E Fluorescent										
🚺 🗹 Photograph	Auto 🤤	Medium	~	8 🔽						
Structure										
🔽 Overlay 🛛	🗹 Overlay 📃 Lights 🗹 Alignment Grid									
Field of View: C	~		Sysl	em Statu	s					
Service 12.9		Idle					Acquire	e		
Service 12.9	cm									
Subject height: 1.50	) 🛟 cm				(		➡ Sequence	Setup		
Focus: use subject	t height 🔽	Temperature:			Locked		Initializ	•		



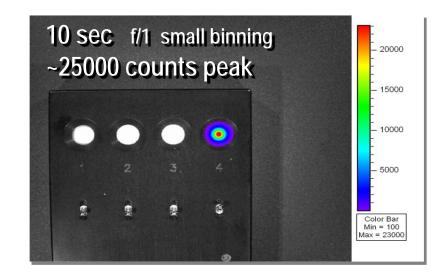
 Signal level is directly proportional to exposure time

Caliper LifeSciences

- Shorter exposure time improves throughput {Recommended min exposure time > 0.5 secs}
- Longer exposure time increases signal {Recommended max exposure time < 5 mins}</li>



💋 IVIS Acq	uisition C	iontrol F	Panel				
Imaging Mode	Exposure Time	Binning	F/Sto	i <b>p</b> Ex	citation Filter	Emission Filter	
📃 🗹 Luminescent	1.00 🗘 sec	💌 Medium	✓ 1	🖌 Blo	ock	🗸 Open	*
📕 🗌 Fluorescent							
🚺 🗹 Photograph	Auto 🤤	Medium	<b>∨</b> 8	~			
Structure							
🗹 Overlay	📃 Lights 🗹 A	lignment Grid					
Field of View: C	*		System S	tatus			
Service 12.9	cm	Idle			[	Acqu	ire
Subject height: 1.50	D 😂 cm					Sequence	e Setup
Focus: use subjec	t height 🔽	Temperature:		Lo	ocked	Initial	ize



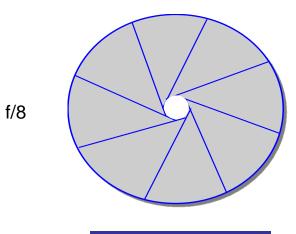
#### Software – Acquisition

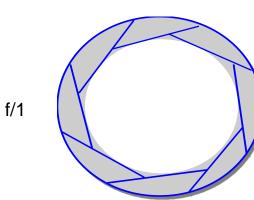


# f/stop (lens aperture)

- 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

💋 IVIS Acqu	uisition (	iontrol P	ar	el				
Imaging Mode	Exposure Time	Binning		F/Stop	Excitation Filter		Emission Filter	
📃 🗹 Luminescent	1.00 🜲 sec	🗙 Medium	~	1 🔽	Block	$\mathbf{v}$	Open	~
Eluorescent				1 2				
🚺 🗹 Photograph	Auto 🤤	Medium	~	4 8				
Structure				U				
🗹 Overlay 🛛	🗌 Lights 🗹 A	lignment Grid						
Field of View: C	*		iys	tem Statu	5			
Service 12.9	cm	Idle					Acquir	e
Subject height: 1.50	r 🛟 cm						✤ Sequence	e Setup
Focus: use subject	: height 🔽	Temperature:			Locked		Initializ	e



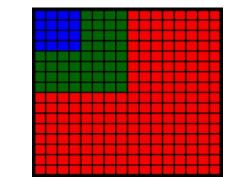




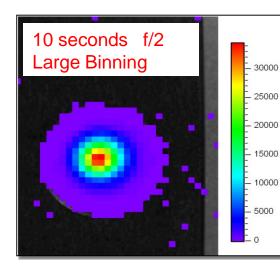
### **Pixel Binning (CCD Resolution)**

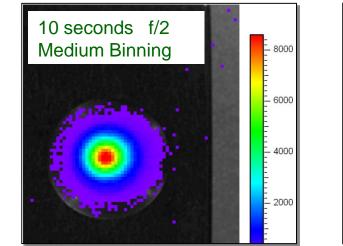
Binning refers to the grouping of pixels into a larger super-pixel

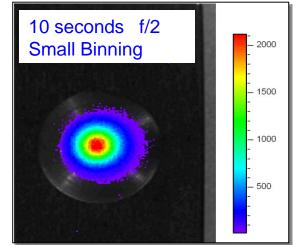
- Large Binning (16) Higher Sensitivity/ Lower Resolution
- Medium Binning (8)
- Small Binning (4) Higher Resolution / Lower Sensitivity



💋 IVIS Acquisitio	n Cor	ntrol P	ane	el					
Imaging Mode Exposure	Time	Binning		=/Stop	Excitation	n Filter	Emission	Filter	
💶 🗹 Luminescent 🛛 1.00 🤤	sec 💌	Medium	~	1	Block	~	Open	~	
📕 📃 Fluorescent		Large Medium							
LI ✓ Photograph Auto ♦		Small	_	8	•				
🖌 Overlay 📃 Lights	🛃 Alignr	ment Grid				_			
Field of View: C 🛛 🔽		9	ōyste	em Stal	us				
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Focus: use subject height	<ul> <li>Ten</li> </ul>	mperature:			Locked			Initialize	





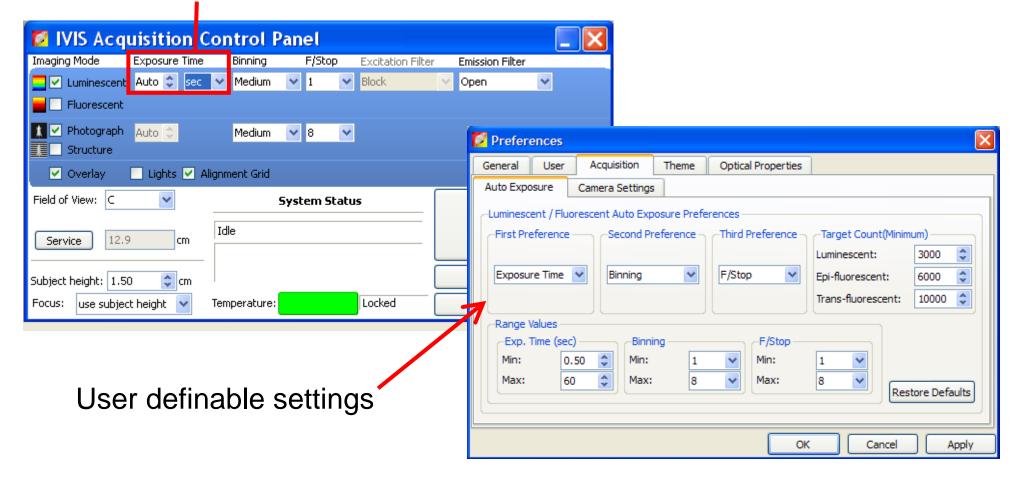


Software – Acquisition



### Auto-exposure

# Auto-exposure feature available for bioluminescence and fluorescence





### **Summary of Basic Camera Settings**

**Controls Sensitivity** 

🜠 IVIS Acqu	lisition Col	ntrol Pan	eļ			
Imaging Mode	Exposure Time	Binning	F/Stop	Excitation Filter	Emission Filte	er
📃 🗹 Luminescent	1.00 🛟 sec 💌	Medium 🔽	1 🔽	Block	🔽 Open	~
Eluorescent						
🚺 🗹 Photograph	Auto 🤤	Medium 🔽	8 💌			
Structure						
🗹 Overlay 🛛 [	🗌 Lights 🗹 Alignr	nent Grid				
Field of View: C	~	Syst	em Statu:	s (		
Service 12.9	cm	le			Acc	quire
Subject height: 1.50	🔹 cm			(	♥ Seque	nce Setup
Focus: use subject	: height 🔽 🛛 Ten	nperature:		Locked	Init	ialize



## Acquisition – Single Image

Overlay will automatically take Photo + Luminescent

💋 IVIS Acq	uisition (	Control P	Panel							
Imaging Mode	Exposure Time	Binning	F/Stop	Excitation Filte	r Emission Filter					
📃 🗹 Luminescent	1.00 😂 sec	💙 Medium	✓ 1	🖌 Block	💟 Open	~				
Eluorescent										
🚺 🗹 Photograph	Auto 😂	Medium	♥ 8	~						
Structure				_						
🔽 Overlay 🗌 Lights 🔽 Alignment Grid										
Field of View: C	*		System Sta	atus						
Service 12.9	9 cm	Idle			Acqui	re				
Subject height: 1.5	0 🛟 cm				* Sequenc	e Setup				
Focus: use subjec	t height 🔽	Temperature:		Locked	Initiali	ze				
			Singl	e Image A	cquisition					
					Software – Acquisition					

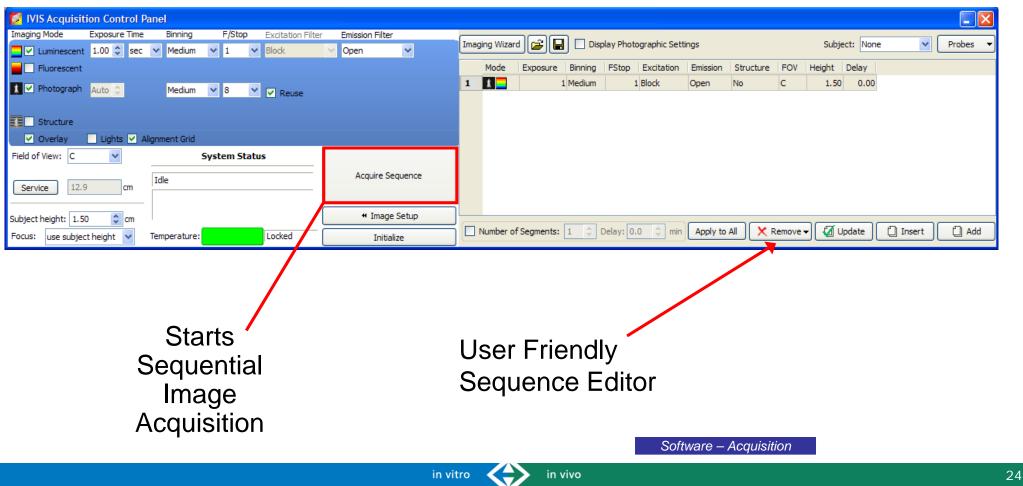
in vitro



## 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)





## **Imaging Wizard**

### Select for assistance in setting up bioluminescence or fluorescence sequences **IVIS Acquisition Control Panel** Imaging Mode Exposure Time Binning F/Stop Excitation Filter Emission Filter

		. /	Excitation inter	Embolorrineer		Impaine Misped
Luminescent 1.00 💲	sec 💉 Medium	✓ 1 ✓	Block 💊	Open	*	Imaging Wizard
Fluorescent						
🚺 🗹 Photograph 🛛 Auto 💲	Medium	✓ 16	Reuse			
🗸 Overlay 🗌 Light	S					1
Field of View: D		System Status	5			
12.5	cm			Acquire Sec	luence	
Subject height: 1.50	cm			Image S	Setup	
Focus: use subject height	<ul> <li>Temperature</li> </ul>		Locked	Initializ	e	Number of Segm

### 🖉 Imaging Wizard Imaging Mode **Bioluminescence Imaging** Select this option for imaging bioluminescent or Bioluminescence chemiluminescent reporters, such as firefly luciferase, click beetle luciferase, renilla, or bacterial luciferase. Fluorescence -Fluorescence Imaging Select this option for imaging fluorescent proteins, dyes, or nanoparticles in the wavelength range of 450-850 nm. Both epi-illumination (illuminationfrom above) and trans-illumination (from below) modes are available. Cancel Next





## Image Labeling

 Good labeling practices are necessary for effective data browsing

XIC20091113133319_004			UserID: XIC 🗸	
			XIC Y	Living Image Universal
Units: Counts Visplay: Overlay 1 fo		<b>1</b>	Saved Labels: LABELS_	1 🕑 🕼 🗶 🖌
Image #: XIC20091113133319_004 Fri ,Nov 13, 2009 11:34:23 Em Filter=640 , Ex Filter=Block	User: SJ Experiment: BLI - DLIT Comment1: Orthotopic PC3M Model		Check any 5 fields for di	
Bin:(HR)16/4, FOV:12.9, f1, 10s Camera: IS0748N4424, Spectral Instruments TE	Time Point: 10mins post injection		🗹 User	SJ 💌
			Group	<b>•</b>
	ENGINE CONTRACTOR		Experiment	BLI - DLIT
		Luminescence		Orthotopic PC3M Model
		L 100	Comment1	
1 - 1		E 80		
		-		
		<u>_</u> 60	Comment2	
		L 40		
		E I	Time Point	10mins post injection
		L 20 Counts	Animal Number	✓
	N.		Animal Strain	▼
		Color Scale Min = 19	Animal Model	✓
		Max = 108		

### Software – Cataloging

Edit Image Labels



 $\mathbf{x}$ 



### Image Cataloging & Browsing Tools

Living Image® Brows	er								
CK20090330114556_SEQ	Click Number	EX Fil	EM Fil	Illumination Mode	Use	User	Group	Experiment	Cor 🔺
					СК		XF750 pillows made 7/31/08	1.9e13/uL, 2:1 dilution, 2uL each	XF7
	⊕- <b>5E</b> CK20080731155429_SEQ				СК	745/800	XF750 pillow in rod made 7/31/08	9.5e12/uL, 2uL each	XF7.
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	⊕- <b>5E</b> QCK20090330120010_SEQ				СК			PC3M-tdT Prostate Ortho Avastin-750 injection	Mou
	⊕ <b>€_0</b> TLT20050624145507_SEQ				TLT				
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	<								>
Hide Browse View	Close Preview Label Set: All			Add to List Brow			Default Configure		Close
	Location: C:/Documents and Settings/jor	iess/De	sktop/X	enogen/Training m	ateria	al/LI4 imag	es/DLIT + FLIT/Avastin-AF-750 on	PC3M-tdTomato/CK20090330114556_SEQ/Sequ	enceInfo .::

**User defined information** 

Software – Cataloging

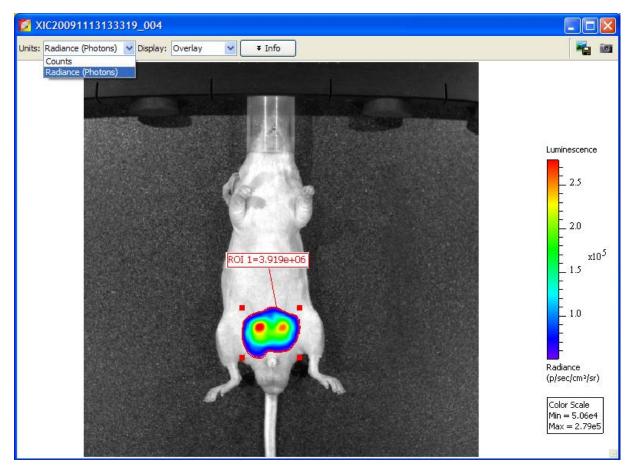






Caliper

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



Tool Palette	×
🗸 Image Adjust	6
< <	9
~ Photo Adjustment	- ^
Brightness: 100	
Gamma: 1.5	
Opacity: 100 🗧	
Color Scale	Ξ.
Min: 5.28e7 💲	
Max: 2.19e8 💲	
Color Scale Limits	
🔾 Auto i 🔿 Full 💿 Manual	
Individual	
Color Table	
Reverse 🗌 Logarithmic Scale	;
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> Image Information	
🖓 ROI Tools	
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Apply to Sequence	
Type: Measurement ROI	<b>~</b>
- Save ROIs	
Name: ROI_2_BNT	Ě
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Threshold %: 10	÷
	× ×

Software - Analysis

in vitro



## **Regions of Interest Tools**

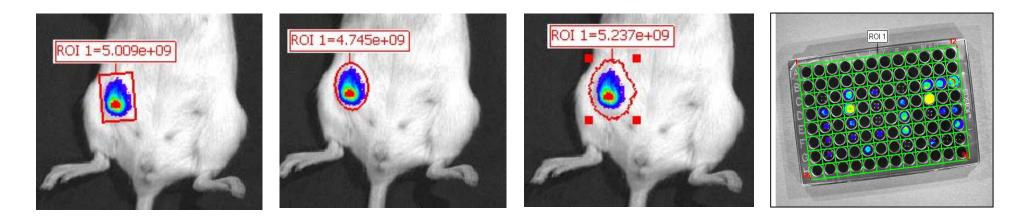
ROI shapes available:

- Square
- Circle
- Contour
- Grid

ROI's can be created:

- Manually
- Automatically
- Free Draw

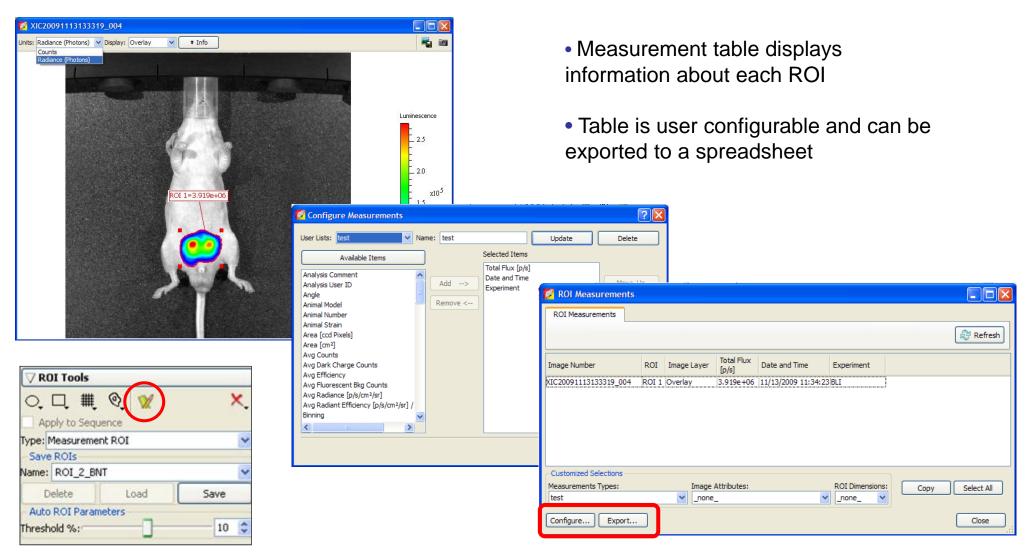
### Important to be consistent with ROI selections





### **Measurement Table**

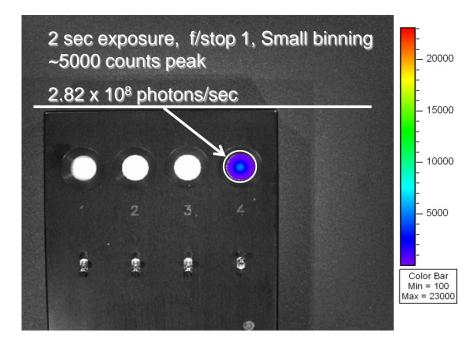
Caliper

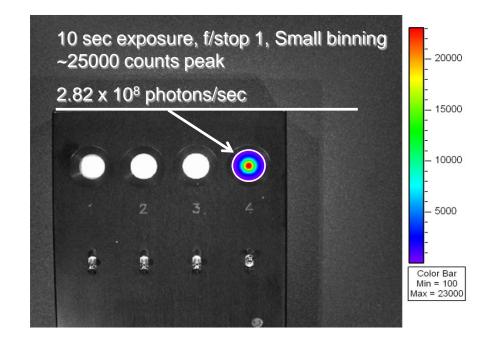




## **Calibrated Physical Units**

- Living Image<sup>®</sup> 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.





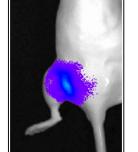


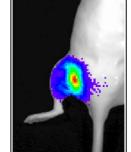


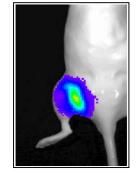
### Calibrated Physical Units vs Raw Signal - Example

Raw Signal (Counts) E B D Peak

400







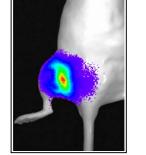
Exp time:		30 sec	30 sec	60 sec	60 sec	60 sec	60 sec
Binning:		small	small	small	small	medium	medium
Day:		1	2	3 4	Ļ	5	6
k Counts	1600 - 1200 - 800 -	-					

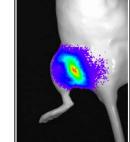


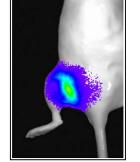
### Calibrated Physical Units vs Raw Signal- Example

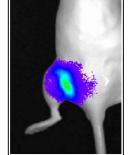
Calibrated Signal

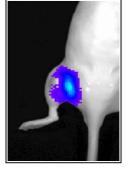
(Photons per second)

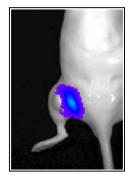












Exp time:	30 sec	30 sec	60 sec	60 sec	60 se	ec 60 sec	
Binning:	small	small	small	small	medium	medium	
Day:	1	2	3	4	5	6	
Radiance: <i>Photons</i> per second	-						

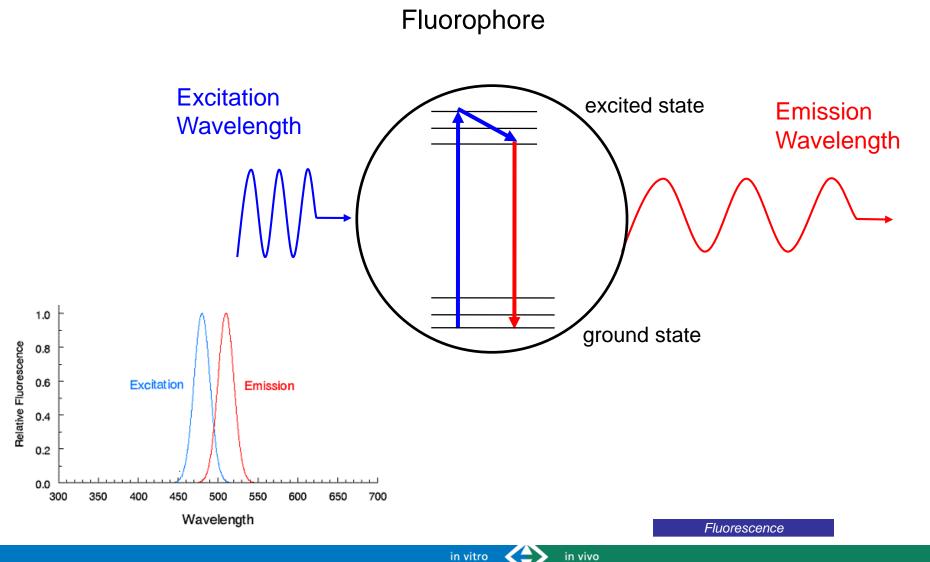
Software - Analysis

in vivo

Sonware - An



### **Fluorescence**

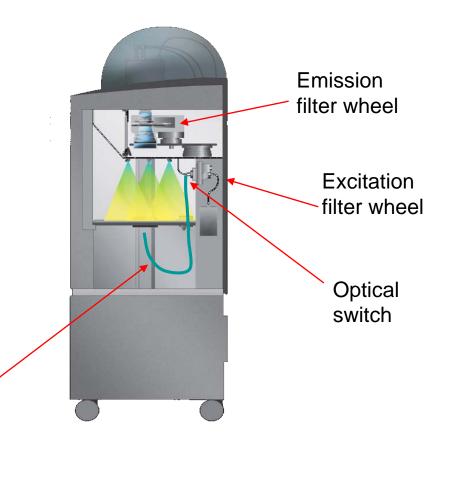




- •18 Emission filters (computer controlled)
- •10 Excitation filters (computer controlled)
- 150 Watt Tungsten/Halogen lamp (computer controlled intensity)

Caliper

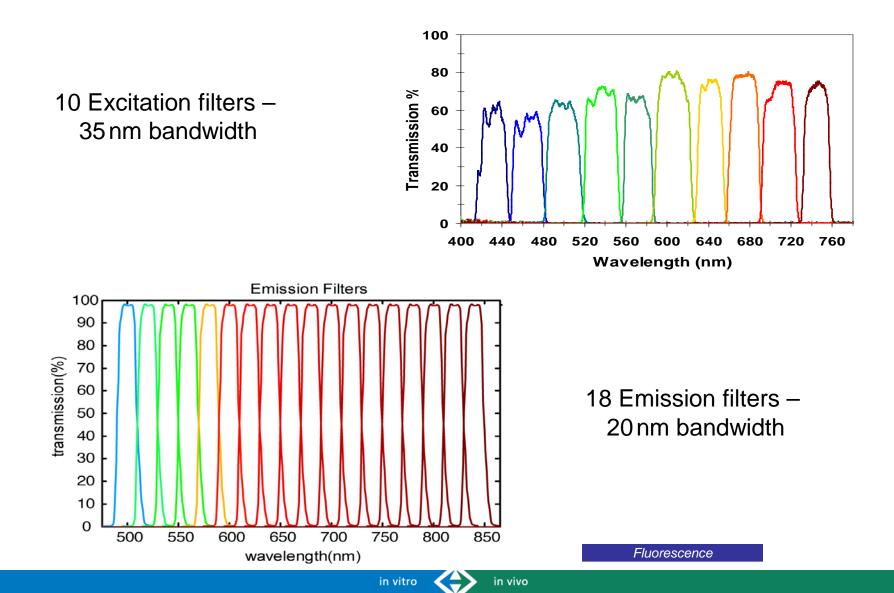
•Low Auto Fluorescence optics and fibers



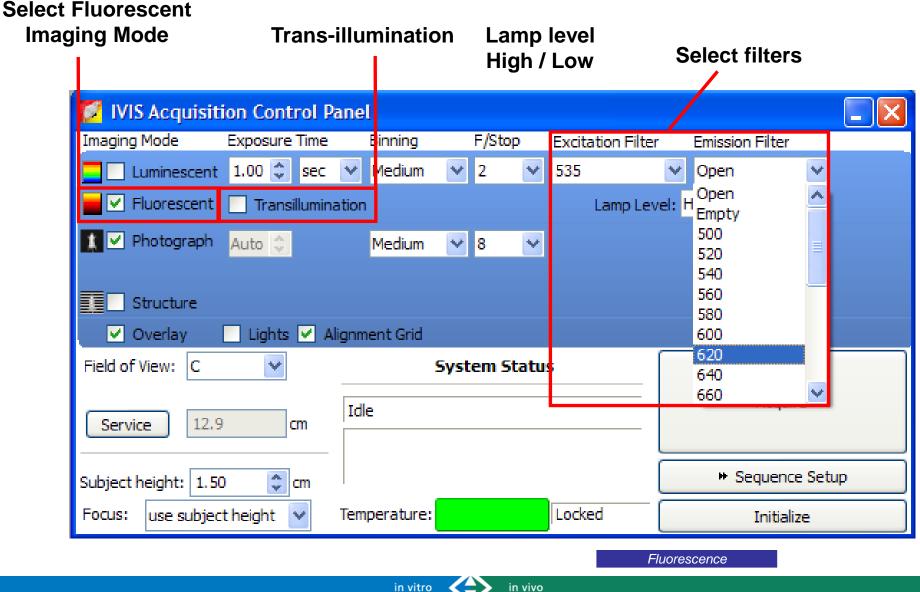
**Trans-illumination** Light source



### **Excitation and Emission Filters**

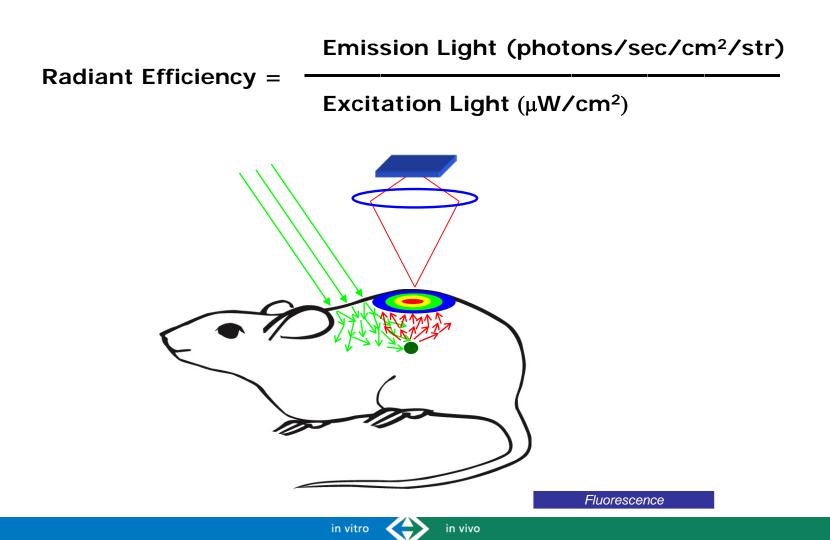








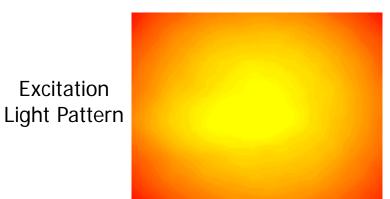
### Fluorescent Calibrated Units: Radiant Efficiency





Counts

### Fluorescent Calibrated Units: Radiant Efficiency

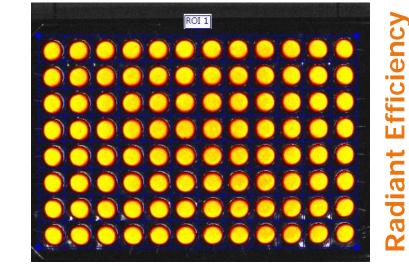


Units of 'Radiant Efficiency' compensates for non-uniform excitation light pattern

#### **GFP Well Plate Uncorrected**

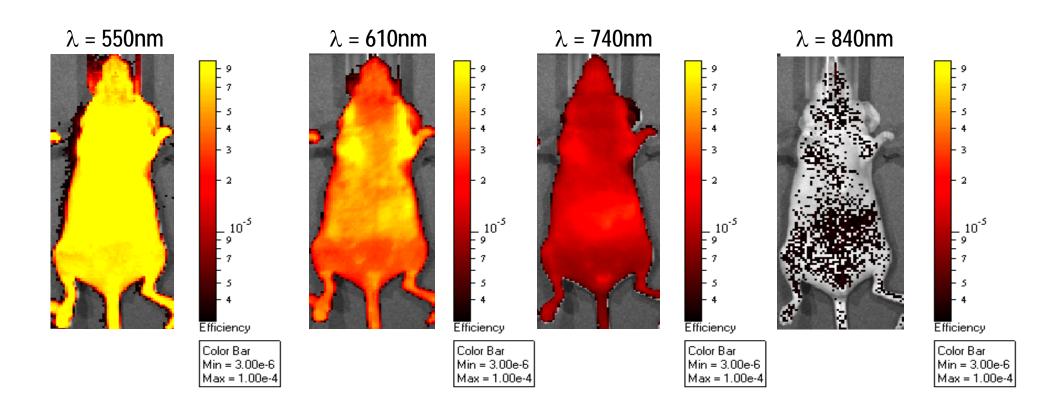
VS.

GFP Well Plate Corrected





### **Auto-fluorescence of Control Mice**







-5

3

2

-5

Efficiency

Color Bar

Min = 3.00e-6

Max = 1.00e-4

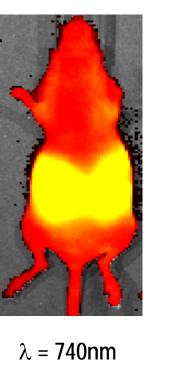
in vitro

in vivo

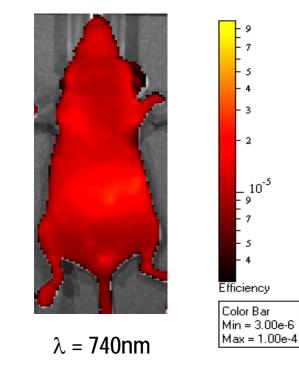
10-5

#### Regular Rodent Food

Caliper



#### Alfalfa Free Rodent Food

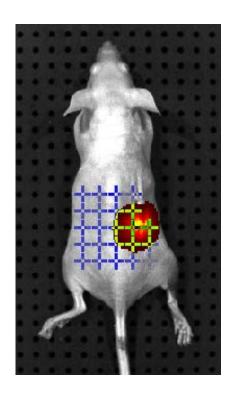




### **Transmission Fluorescence**





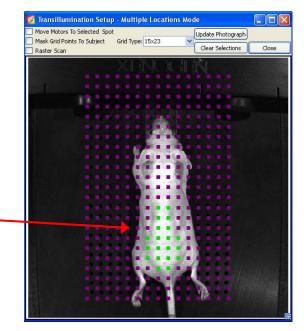




### **Trans-illumination Sequence**

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

Select trans-illumination points

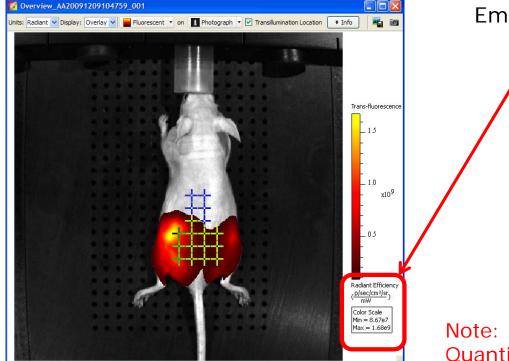


🜠 IVIS Acquisition Control Panel													- 🗙
Imaging Mode Exposure Time Binning F/Stop Excitation Filter	Imaging Wizard 😰 🔲 🗆 Display Photographic Settings						s	Subject: None V Probes V					
□ Luminescent Auto  sec  Medium  2  710 Fluorescent  Transilumination Normalized Setup Lamp Leve	<ul> <li>✓ 800</li> <li>✓</li> <li>✓</li> </ul>			Exposure	Binning		Excitation	Emission Lamp Level	Structure	FOV	Height	Transillumination	De 🔨
I ♥ Photograph Auto ♦ Medium ♥ 8 ♥ ♥ Reuse		1		Auto	Medium	2	710		No	с	_	15x23:19,6	
Reuse	2 1 Auto Medium 2 710 800 High No C 1.50 15x23:19, 7												
			175.	Auto	Medium	2	710		No	С	1.50	15x23:19,8	
✓ Overlay Lights ✓ Alignment Grid			275	Auto	Medium	2	710		No	С		15x23:19,9	
Field of View: C V System Status		_	2015	Auto	Medium	2	710		No	С		15x23:19, 10	
System status			1775	Auto	Medium	2	710		No	С		15x23:18,6	
Service 12.9 cm Idle	Acquire Sequence	_	275	Auto	Medium	2	710		No	С	1.50	15x23:18, 7	
Service 12.9 Cm		8	<u>*</u>	Auto	Medium	2	710	800 High	No	С	1.50	15x23:18,8	~
Subject height: 1.50 🔿 cm	◀ Image Setup									_			>
Focus: use subject height 👻 Temperature:	Initialize	] 🗆 NI	umber of	Segments:	1 🗘 [	Delay: 0.0	) 🌲 min	Apply to All	emove 🗸 🕻	🖞 Updat		Insert 📋	Add



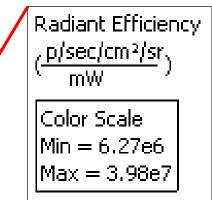


### **Trans-illumination units**



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

Emission Radiance ÷ Excitation light power



Note: Quantification not comparable to Epi-Fluorescence images;

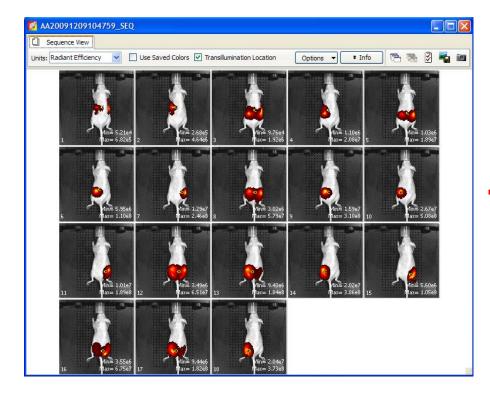
Emission Radiance ÷ Excitation power density (per area)



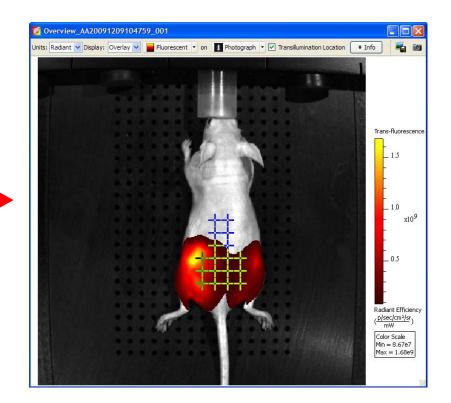


### **Trans-illumination Sequence Overview**

🜠 Living Image® 4.0											
File	Edit	View	Tools	Acquisition	Window	Help					
Ê	٩		Well Plate Quantification for AA20091209104759_SEQ Image Overlay for AA20091209104759_SEQ								
Colorize											
D	Sequen	ce View		Transillumination Overview for AA20091209104759_SEQ Image Math for AA20091209104759_SEQ							



Combined image overview of multiple trans-illumination images





### Advanced topics (preview)

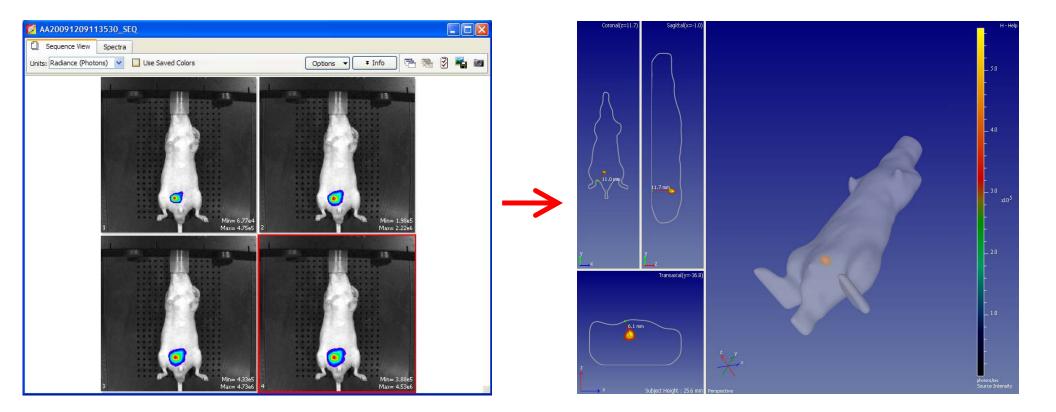
- 3D Tomography (Luminescence and Fluorescence)
- Spectral Un-mixing







### 3D Tomography (Luminescence)

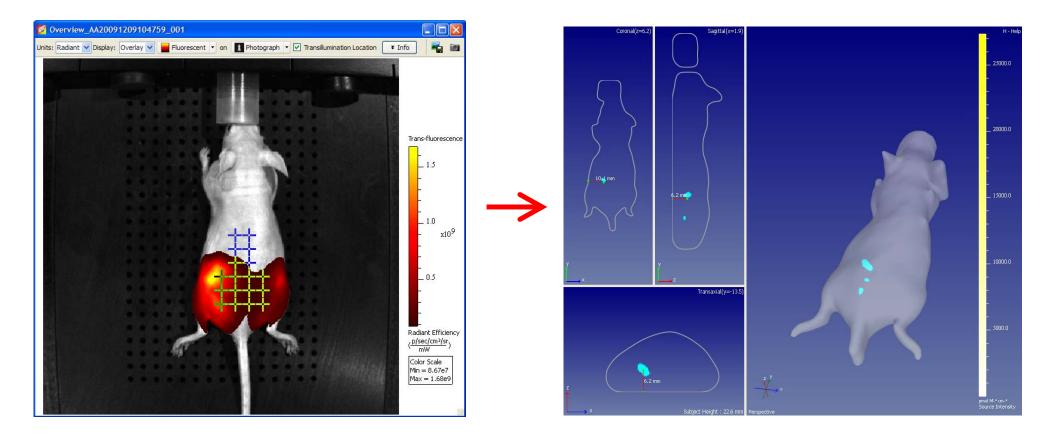


Spectral scans with Emission filters allows for absolute quantification and source localization





### 3D Tomography (Fluorescence)



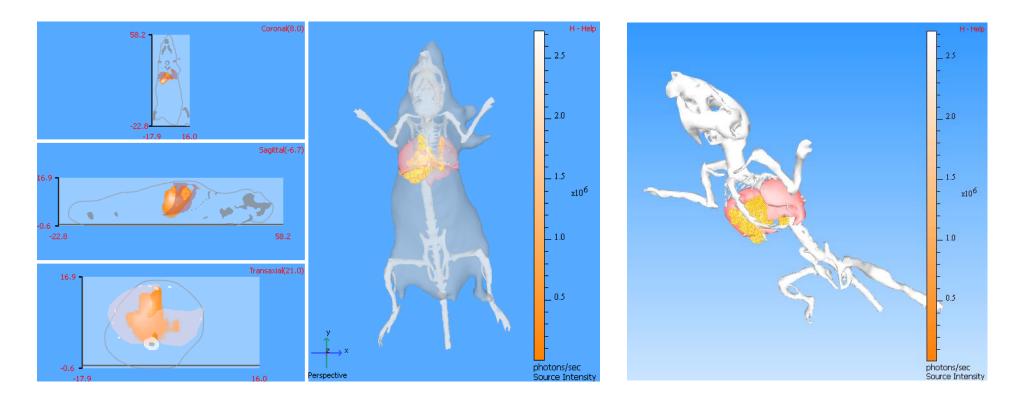
Spatial scans with trans-illumination light source allows for absolute quantification and source localization



🔪 in vivo



# 3D Tomography with co-registration - $\mu$ CT and organ atlas database



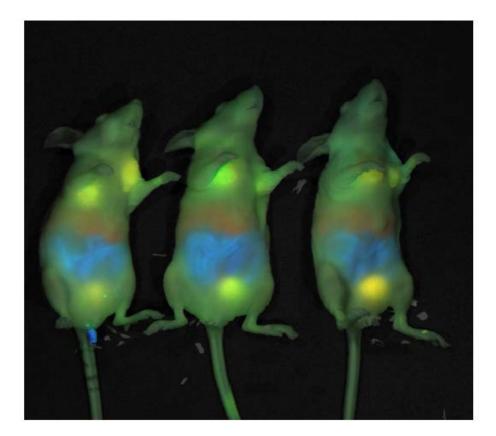
Ability to add and co-register images from a library of  $\mu$ CT / atlas data plus import/export DICOM formatted files





## **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 dependent 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<sup>®</sup> 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

in vivo



### For an In Depth Study

#### **IVIS Software Manual**

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

#### IVIS<sup>®</sup> University

#### Thank you for enrolling in the Caliper IVIS University!

Click on the links below to access the IVIS Blog, Tech-Notes, New Protocols, Feature Updates, and more.

#### IVIS Blog

O Click here to access the IVIS Blog site

#### **Tech-Notes**

Acquisition of High Resolution Images

Determine Areas of Saturation

- Diffuse Light Imaging Tomography (DLIT)
- DLIT Sequence Acquisition

Drawing ROIs

Filter recommendations for common fluorescent proteins, dyes and Quantum Dots using the IVIS Lumina and IVIS
Kinetic equipped with the standard filter set

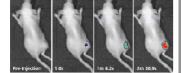
Filter recommendations for common fluorescent proteins, dyes and Quantum Dots using the IVIS Spectrum and Lumina II or IVIS Kinetic equipped with spectral unmixing filters

Software



#### Living Image<sup>®</sup> Software

User's Manual

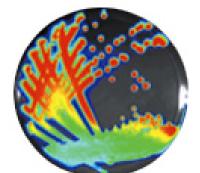


Version 4.0





# **IVIS Bioware and Reagents**



✓ Bioware
 ✓ Bioware Ultra
 ✓ Bioware Ultra Red

### **IVIS** XenoLight



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

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



✓ RediJect D-Luciferin✓ RediJect D-Luciferin Ultra

✓ D-Luciferin Substrate

