IVIS Spectrum: Advanced Small Animal Imaging of Fluorescent and Bioluminescent Probes

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XENOGEN
now part of Caliper Life Sciences

What Will Be Covered?

- IVIS Spectrum features
- New features of Living Image 4.0
- Comparison of Epi and Transillumination
- Normalized transillumination imaging
- Spectral Unmixing
- 3D Reconstructions of bioluminescence (DLIT) and fluorescence (FLIT)
- Well plate quantitation of 3D sources

IVIS® Spectrum

- High sensitivity CCD for bioluminescence or fluorescence imaging
- High throughput with 23 cm field of view
- High resolution (to 20 microns) with 3.9 cm field of view
- 28 filters, wavelength ranges from 490 – 850 nm
- Spectral unmixing using discrete bandpass filters
- Reflection (Epi)- or transmission-mode fluorescence
- Single-view 3D surface topography from structured light
- 3D diffuse tomographic reconstructions for both bioluminescence and fluorescence
- Ideal for imaging multiple probes/reporters

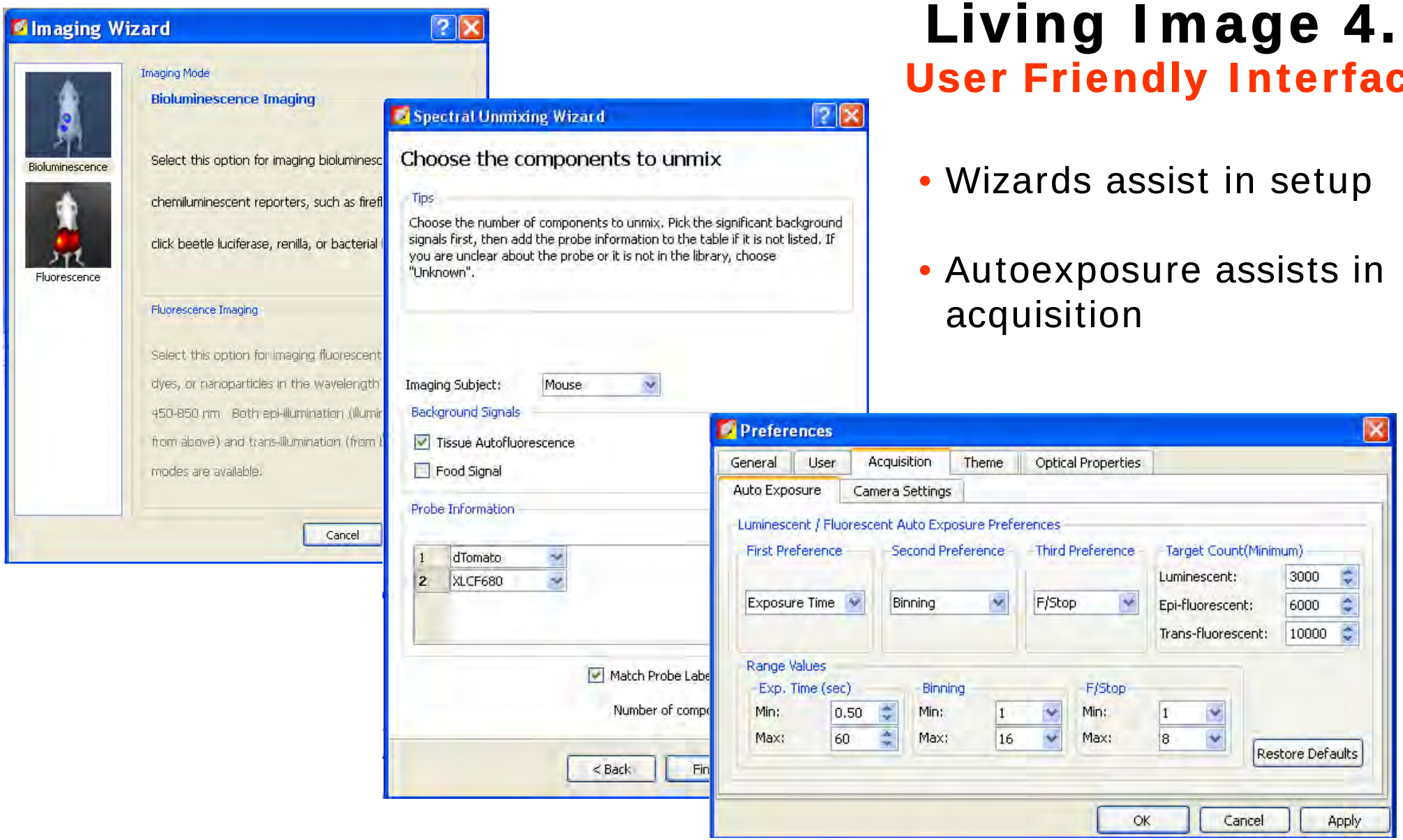
Living Image® Software

- 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

Living Image 4.0

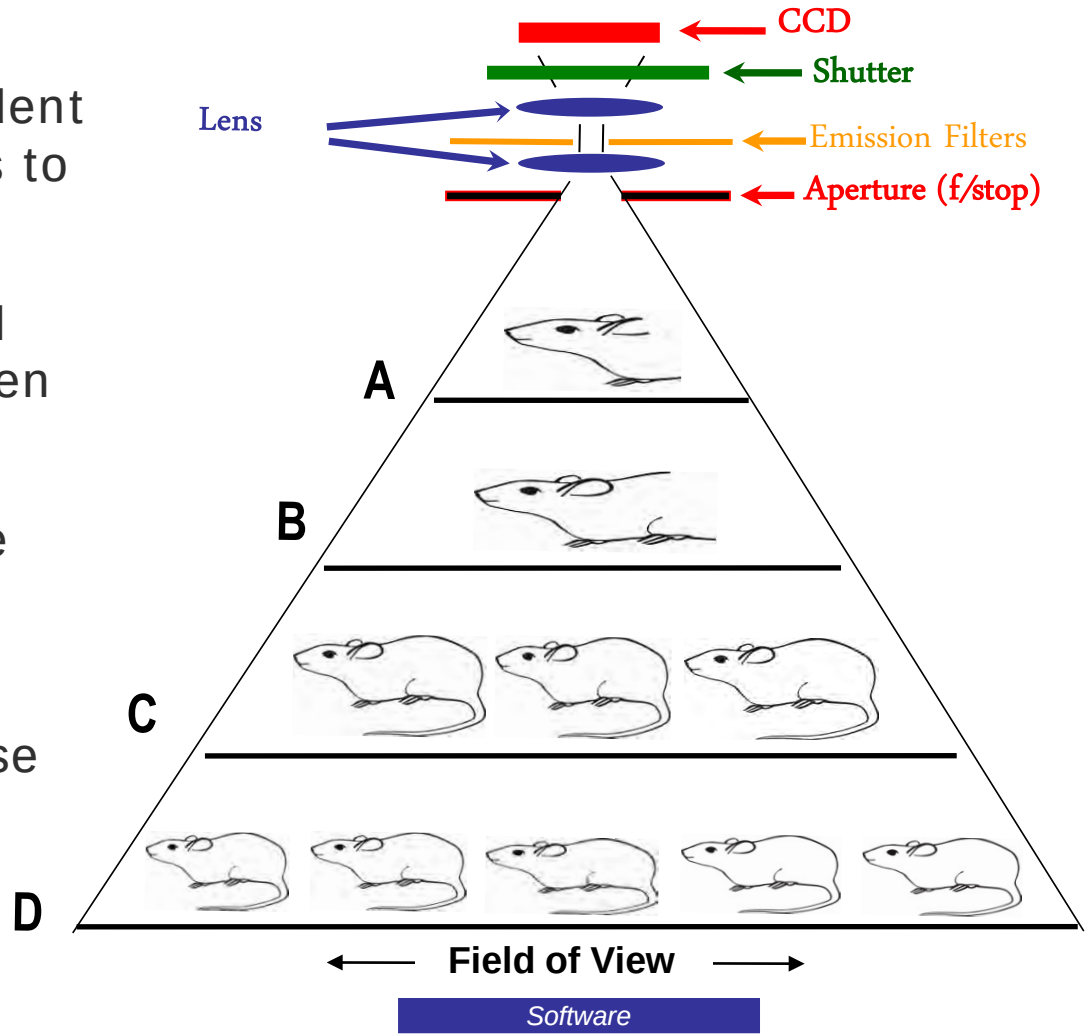
User Friendly Interface

- Wizards assist in setup
- Autoexposure assists in 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

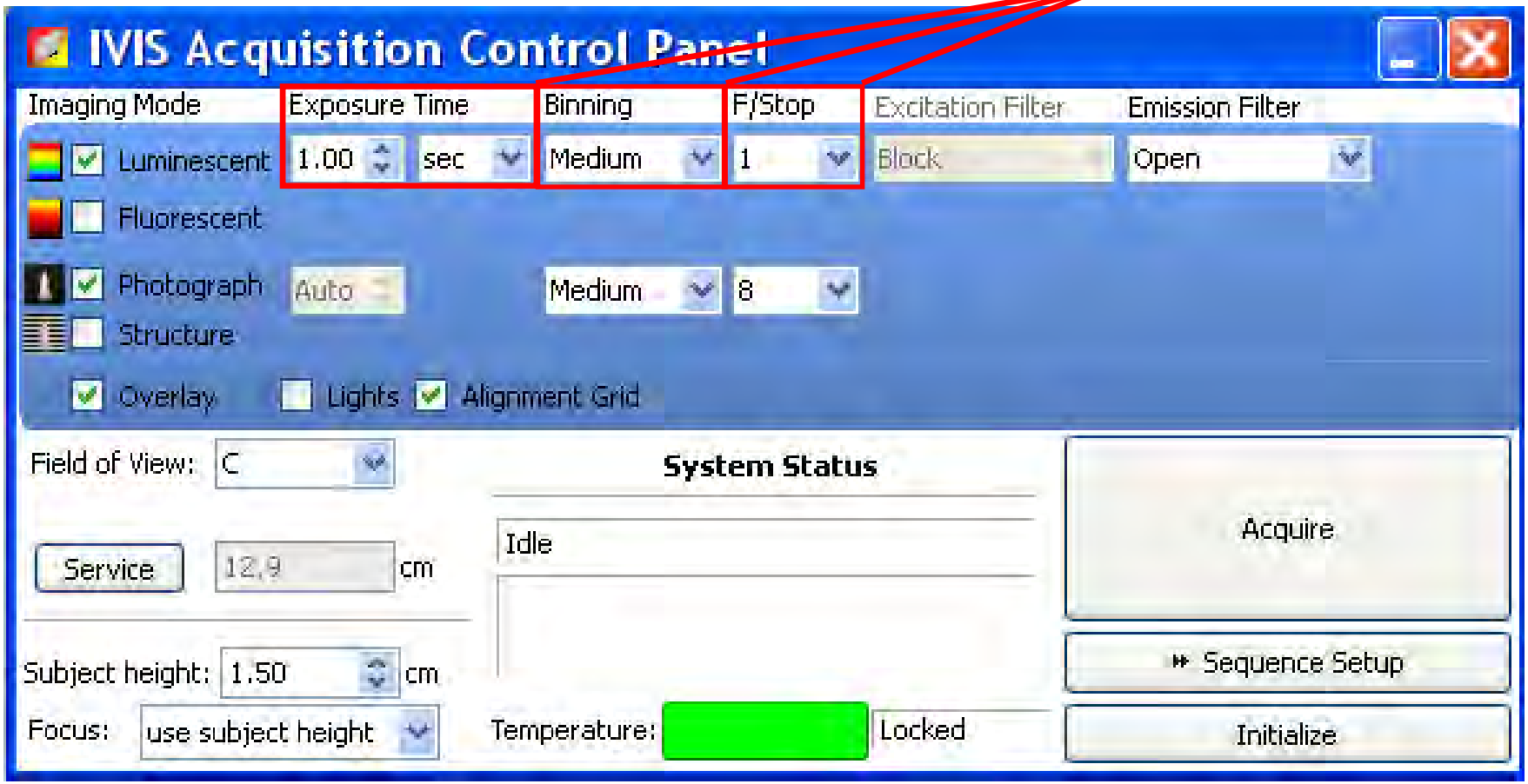


Setting Sensitivity – Luminescent Signal Level

- 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

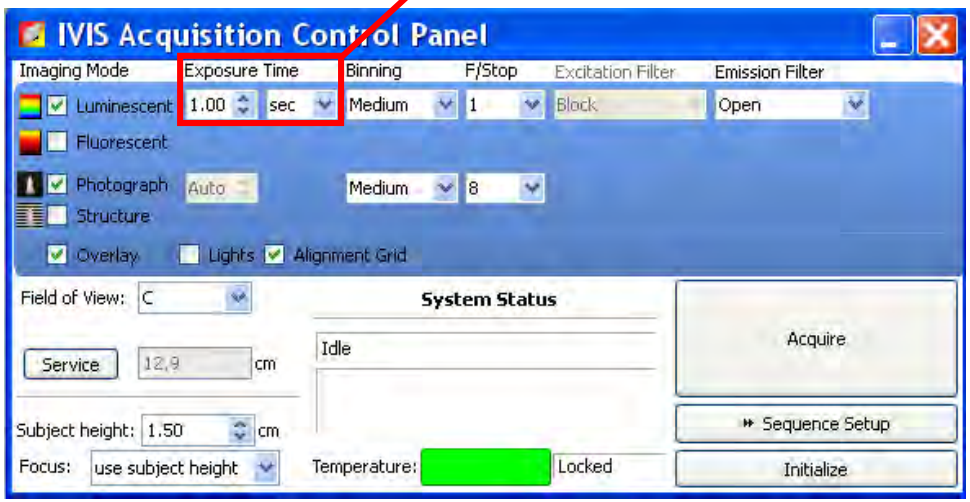


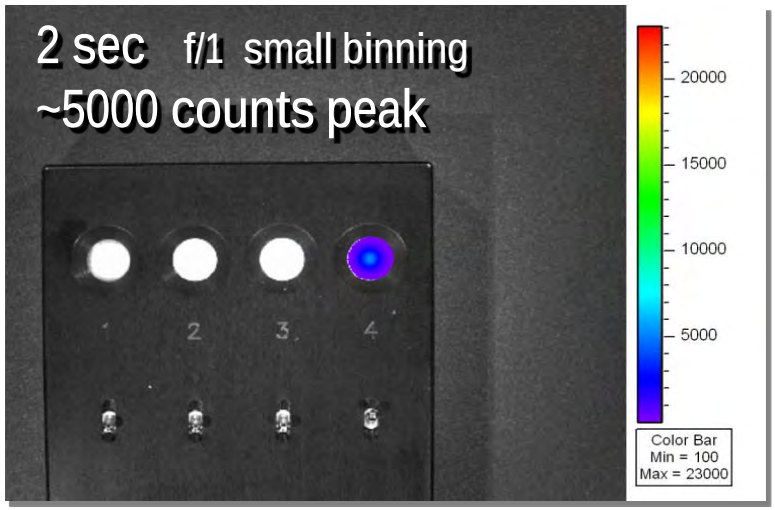
Exposure Time

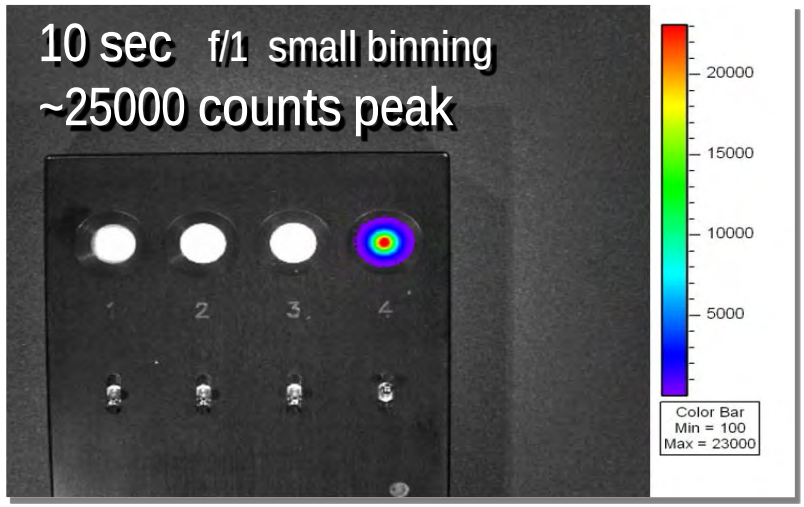
- **Signal level is directly proportional to exposure time**
- **Shorter exposure time improves throughput**

  (Recommended min exposure time > 0.5 secs)
- **Longer exposure time increases signal**

  (Recommended max exposure time < 5 mins)



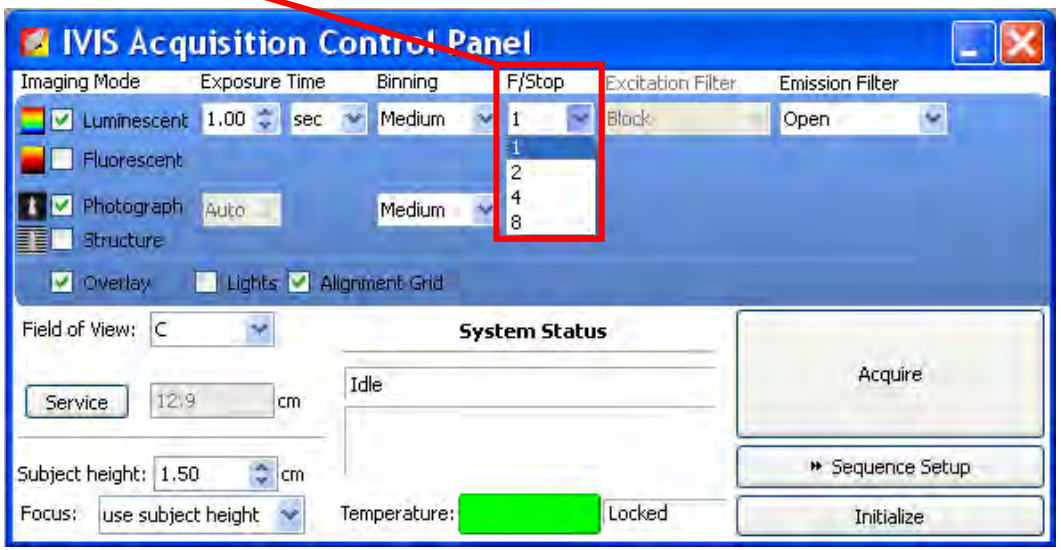




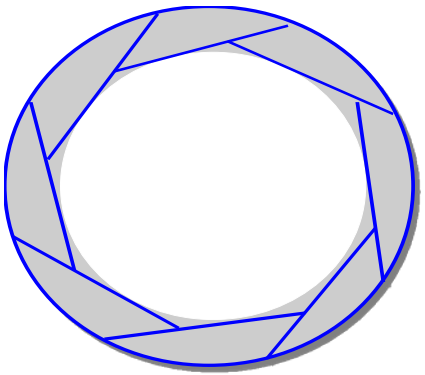
Software – Acquisition

f/stop (lens aperture)

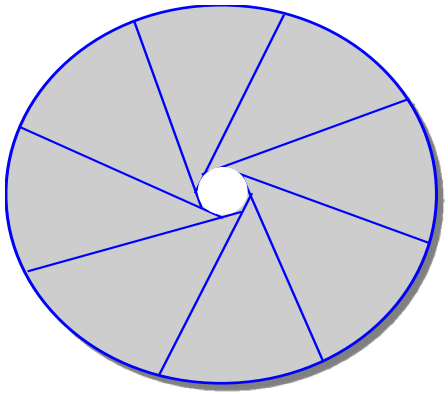
- f/stop controls the amount of light received by the CCD
- f/1 is wide open, maximum light collection - default for luminescent
- f/8 is smallest aperture, best resolution - default for photo
- Changing f/stop changes counts by a factor of 4



f/1



f/8

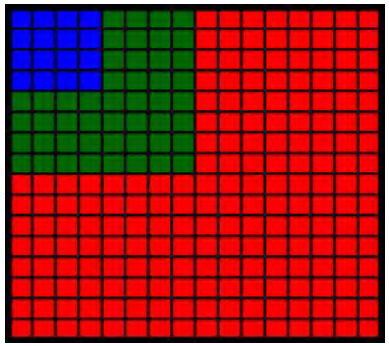


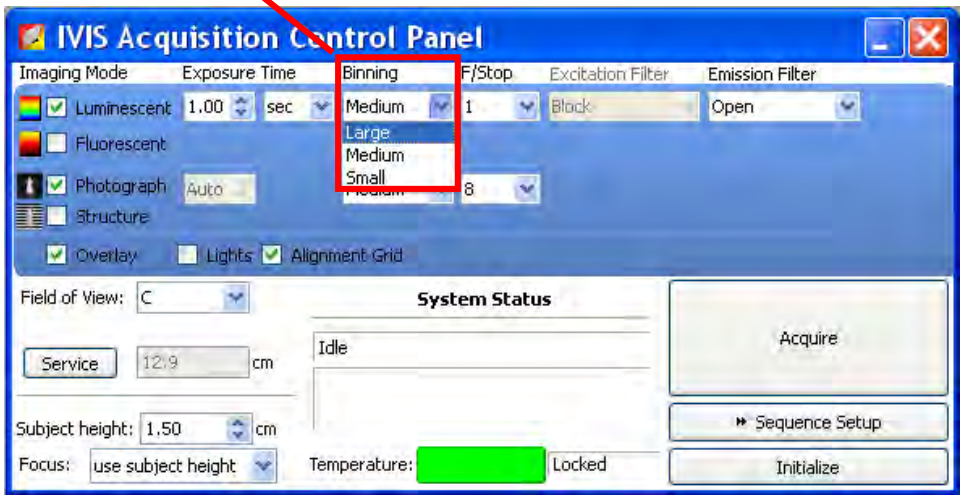
Pixel Binning (CCD Resolution)

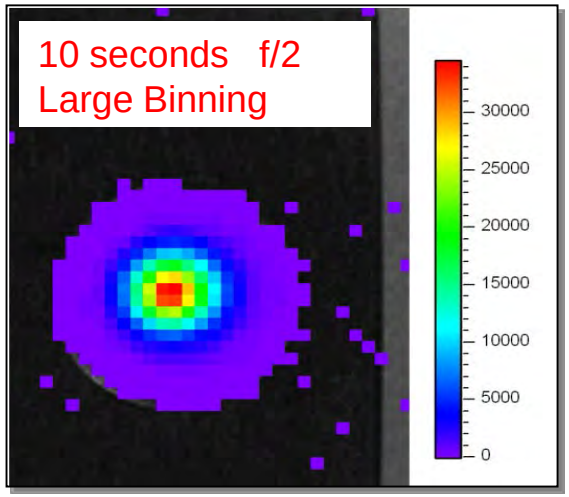
Binning refers to the grouping of pixels into a larger super-pixel

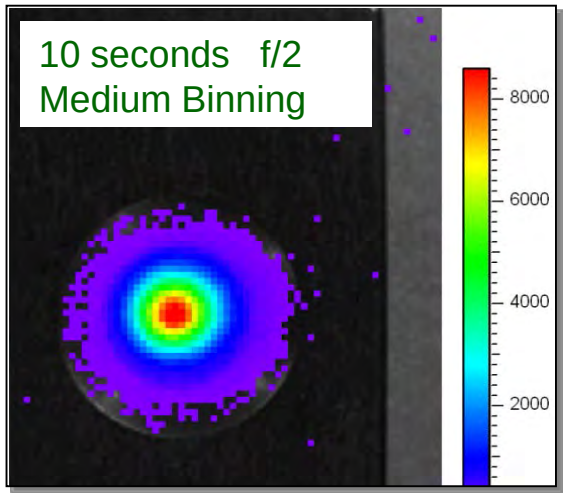
Changing binning settings changes counts by a factor of four

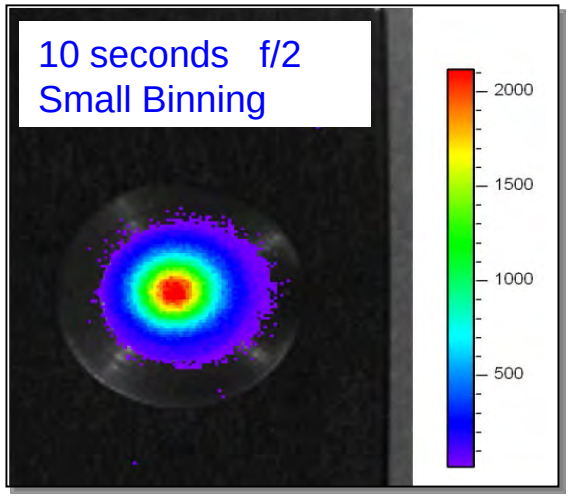
- **Large Binning (16) Higher Sensitivity / Lower Resolution**
- **Medium Binning (8)**
- **Small Binning (4) Higher Resolution / Lower Sensitivity**







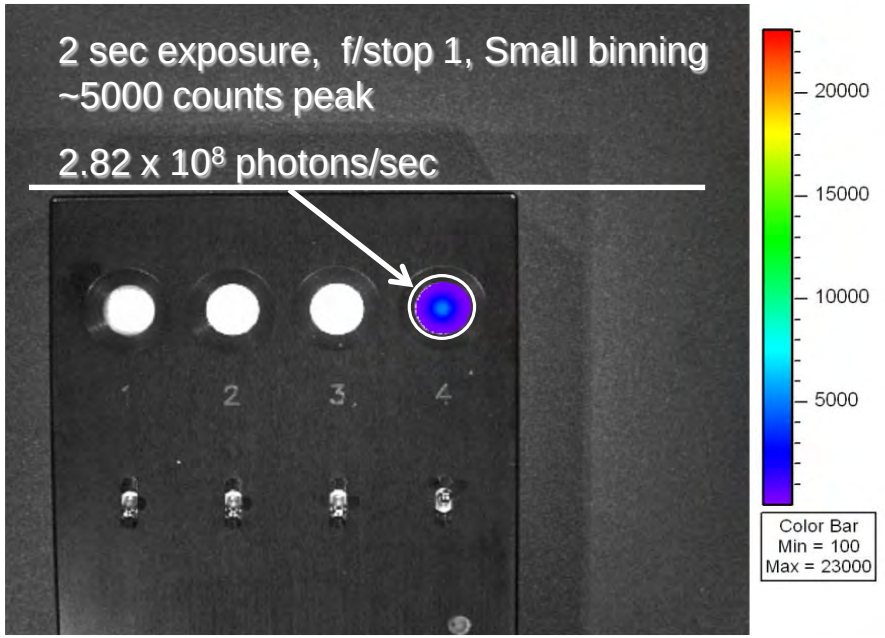


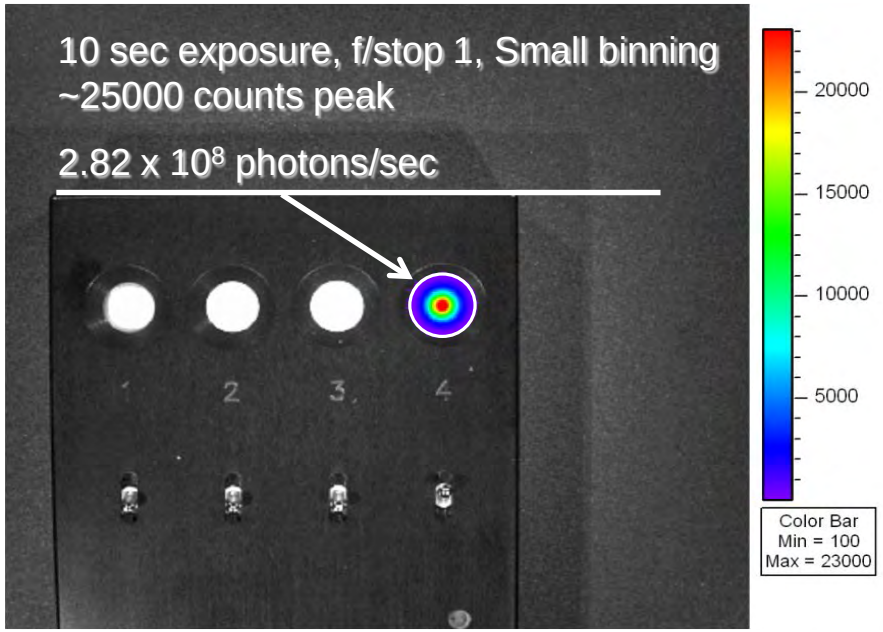


Software – Acquisition

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.

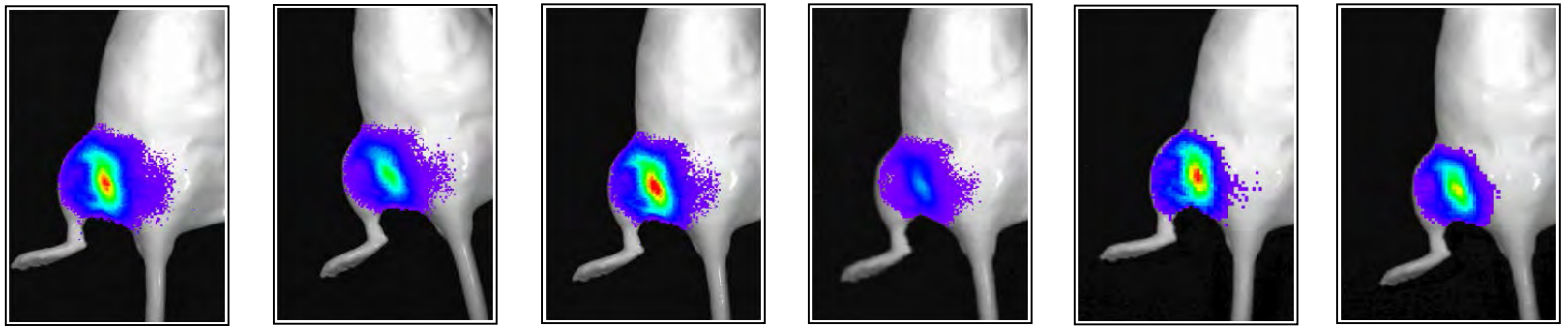




Software - Analysis

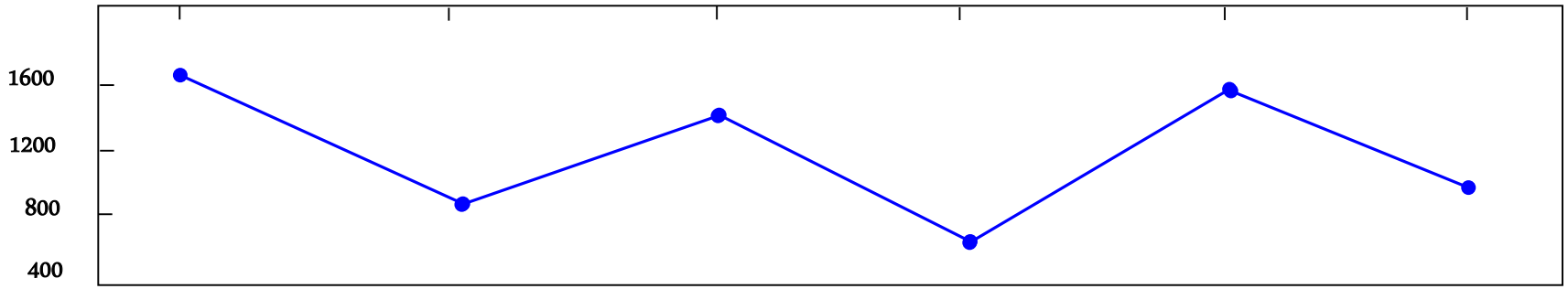
Calibrated Physical Units *vs* Raw Signal - Example

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

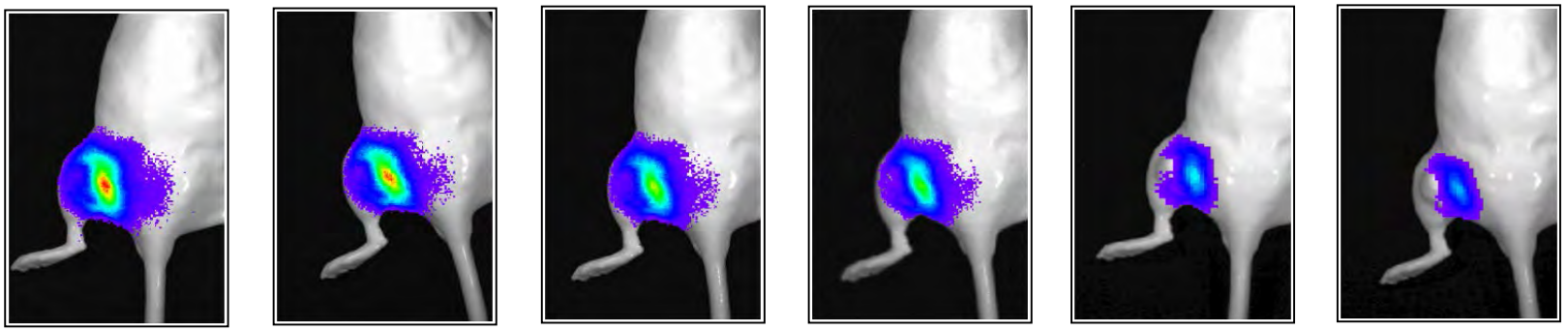


Software - Analysis

Calibrated Physical Units *vs* Raw Signal- Example

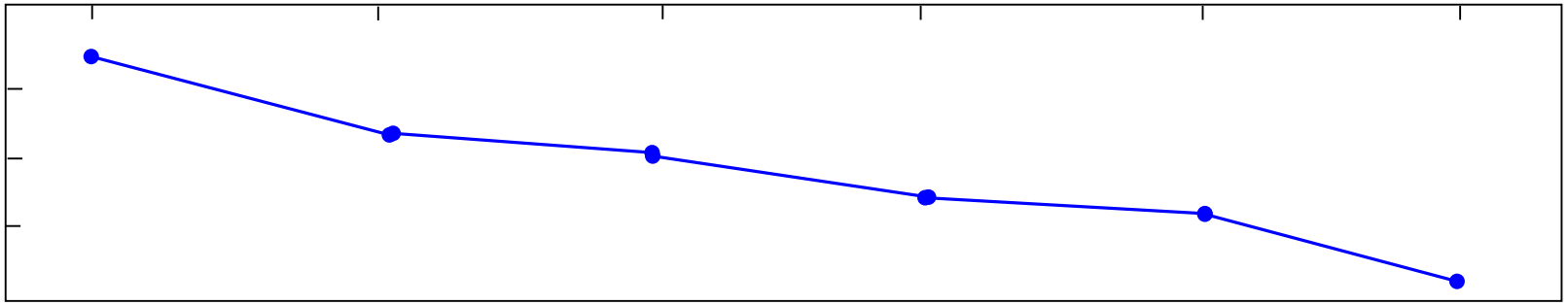
Calibrated Signal

(*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

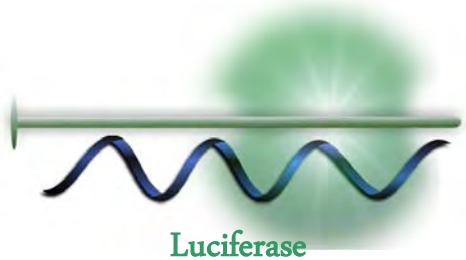
Radiance: *Photons per second*



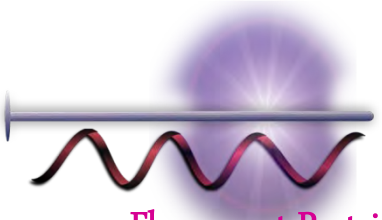
Software - Analysis

Imaging Basics

Reporter Molecules



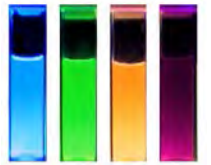
Luciferase



Fluorescent Proteins

Quantum dots and Nanoparticles

Fluorescent dyes



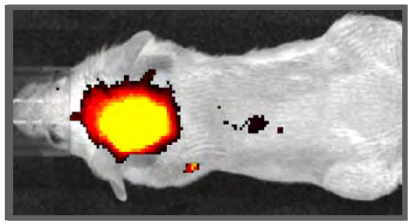
+ ATP and O_2 – Live cells

+ D-luciferin substrate

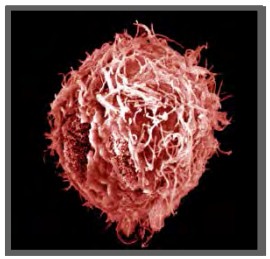
Transfection

Direct cell/protein labeling

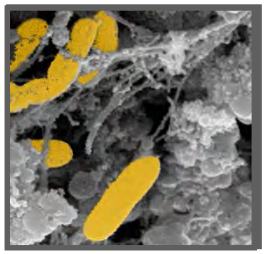
+ excitation light source



Genetic Marker



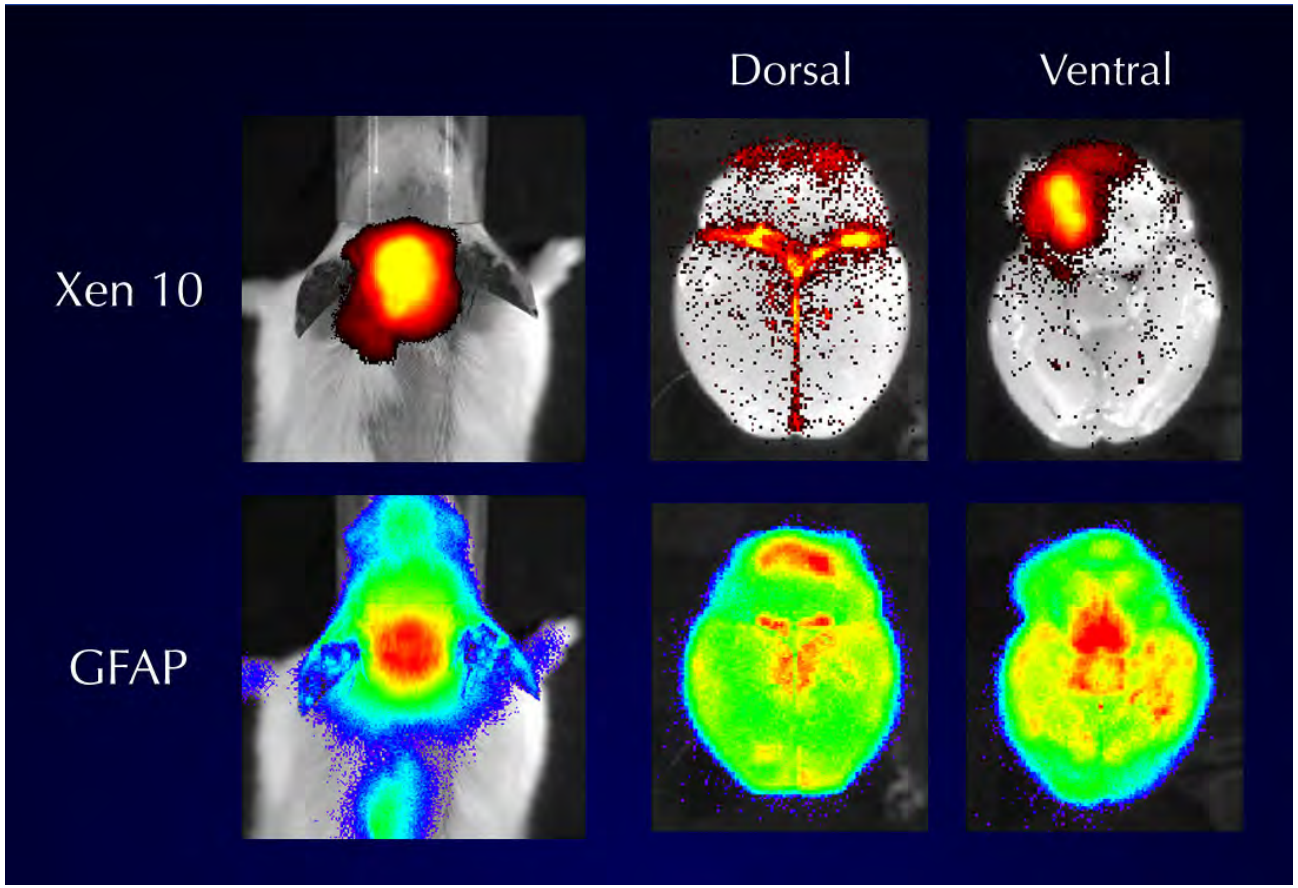
Label Cells



Label Bacteria

Label Proteins

Dual Reporter: Bacterial luc and GFAP Brain Imaging From Mice with Pneumococcal Meningitis



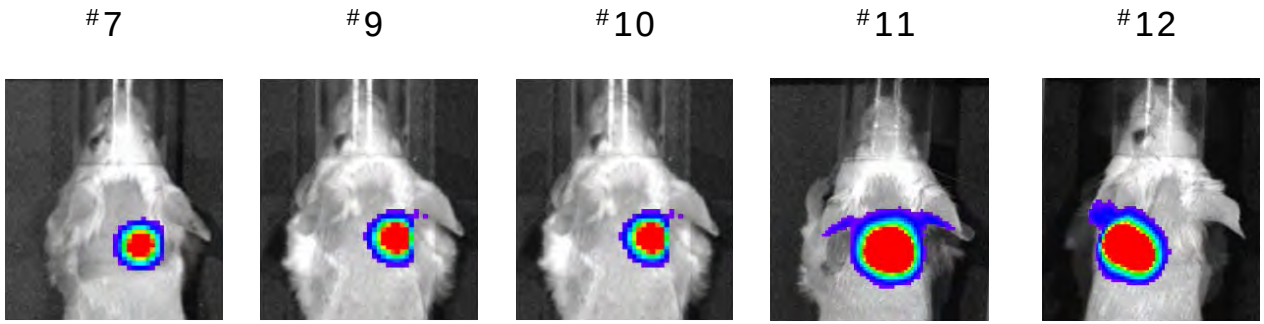
Bacterial luc
~ Open filter

Firefly luc
~ 620 nm

Kadurugamuwa et al., Infection and Immunity, 2005

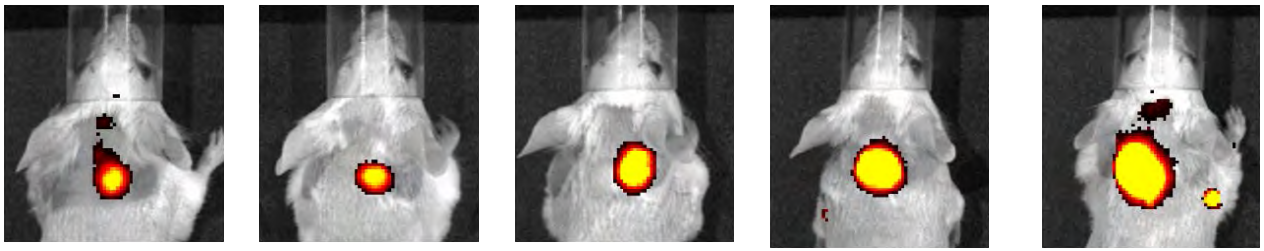
Tumor Imaging with Bioluminescence and Fluorescent HER2 Affibody

Bioluminescent imaging of SKOV3-luc tumor cells



Images were taken after i.p. injection luciferin.

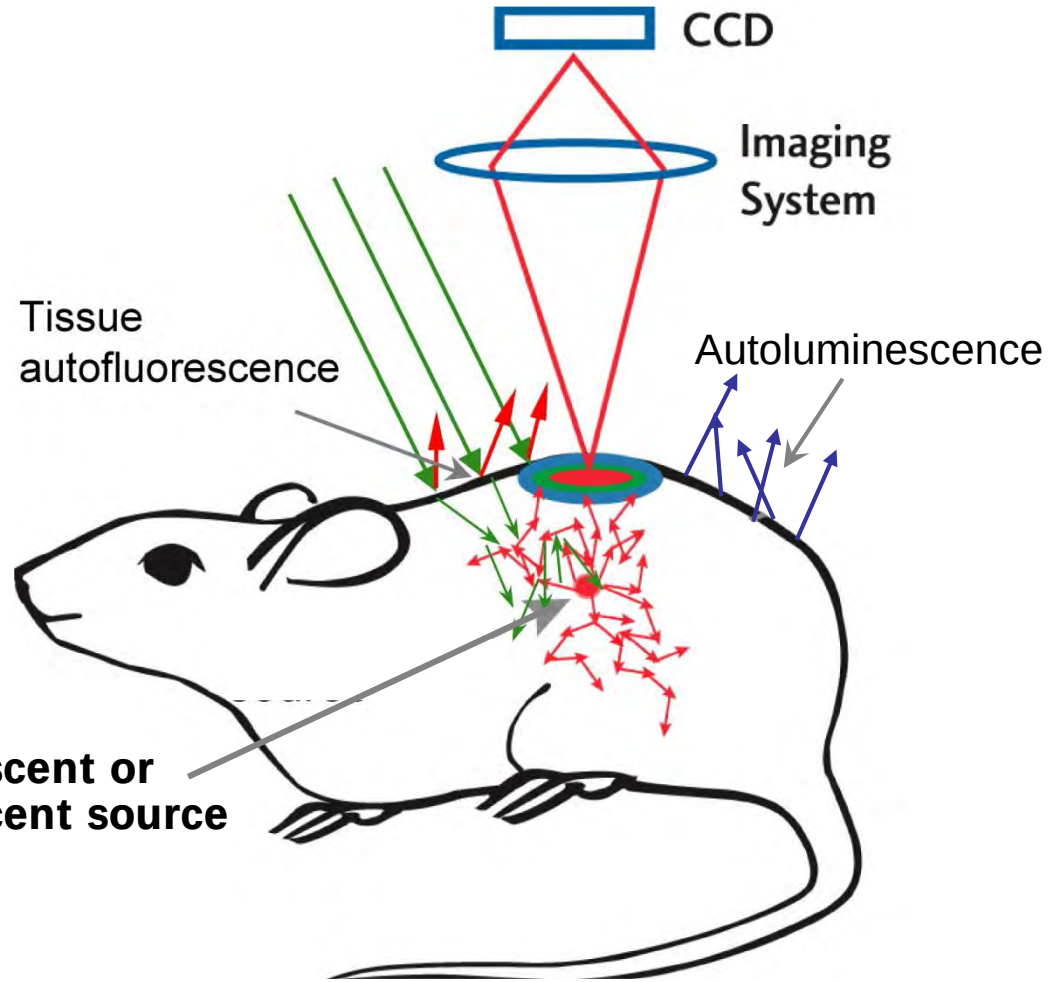
Fluorescent imaging of HER2 affibody labeled with XenoFluor 680



Images were taken at 3 hours after i.v. injection of the HER2/XF680 affibody probe

Challenge of *In Vivo* Optical Imaging

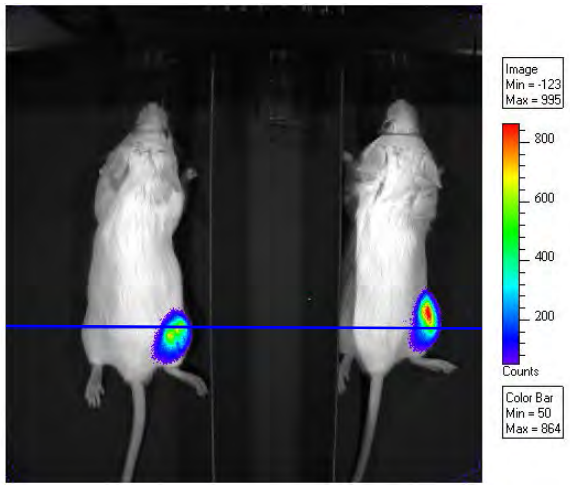
- Photons are absorbed and scattered in tissue
- Surface signal depends on source depth
- Tissue is both autoluminescent and autofluorescent
- Autofluorescence levels are much higher than autoluminescence

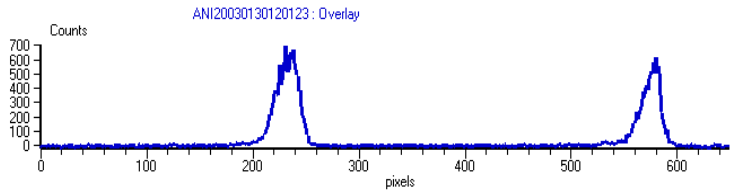


Sensitivity is a function of Signal to Noise

Luminescent Sources:
Signal brightness generally lower than fluorescent sources

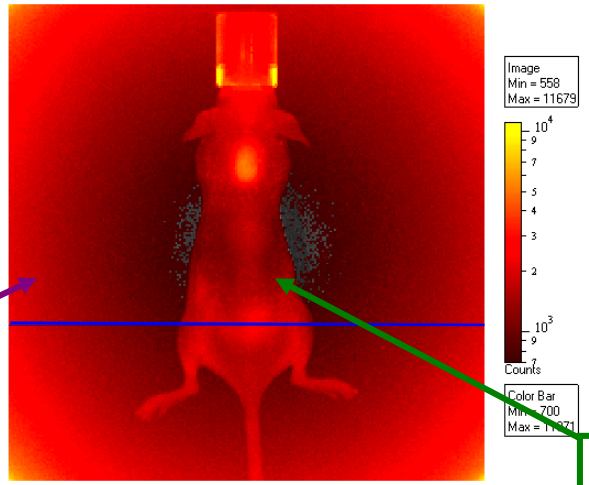
Higher sensitivity due to low level noise: both instrument and animal autoluminescence





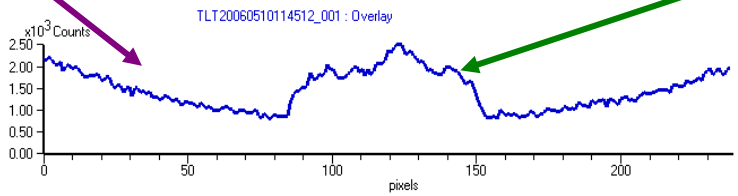
Fluorescence Sources:
Signals generally brighter than luminescent sources

Lower sensitivity due to higher noise: instrument background and autofluorescence



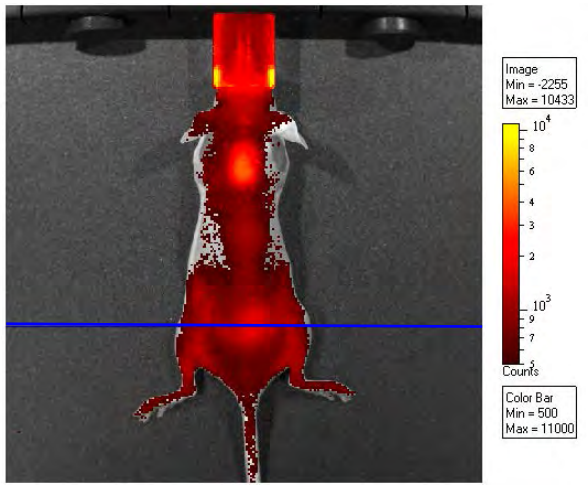
Instrument Background

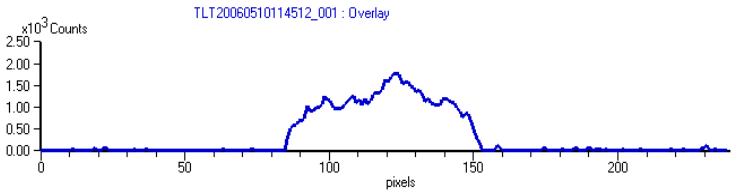
Autofluorescence



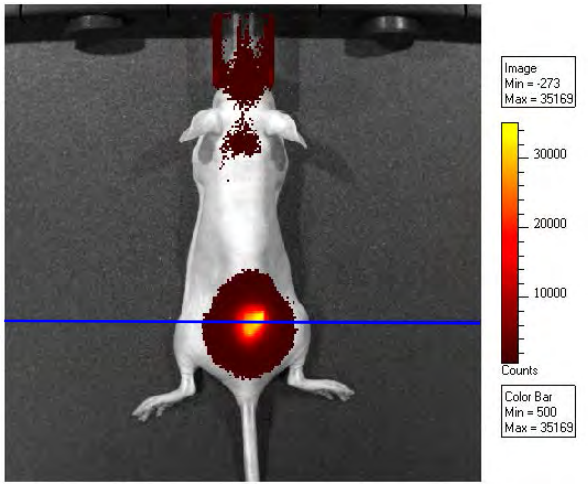
Improvements to Signal to Noise Ratio

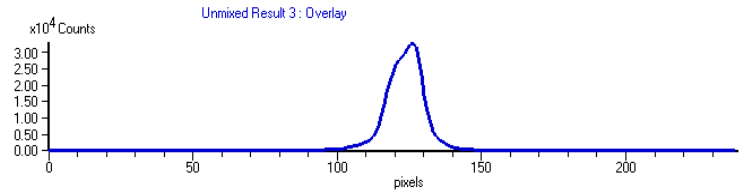
Adaptive FL Background Subtraction: Software tool to reduce instrument background



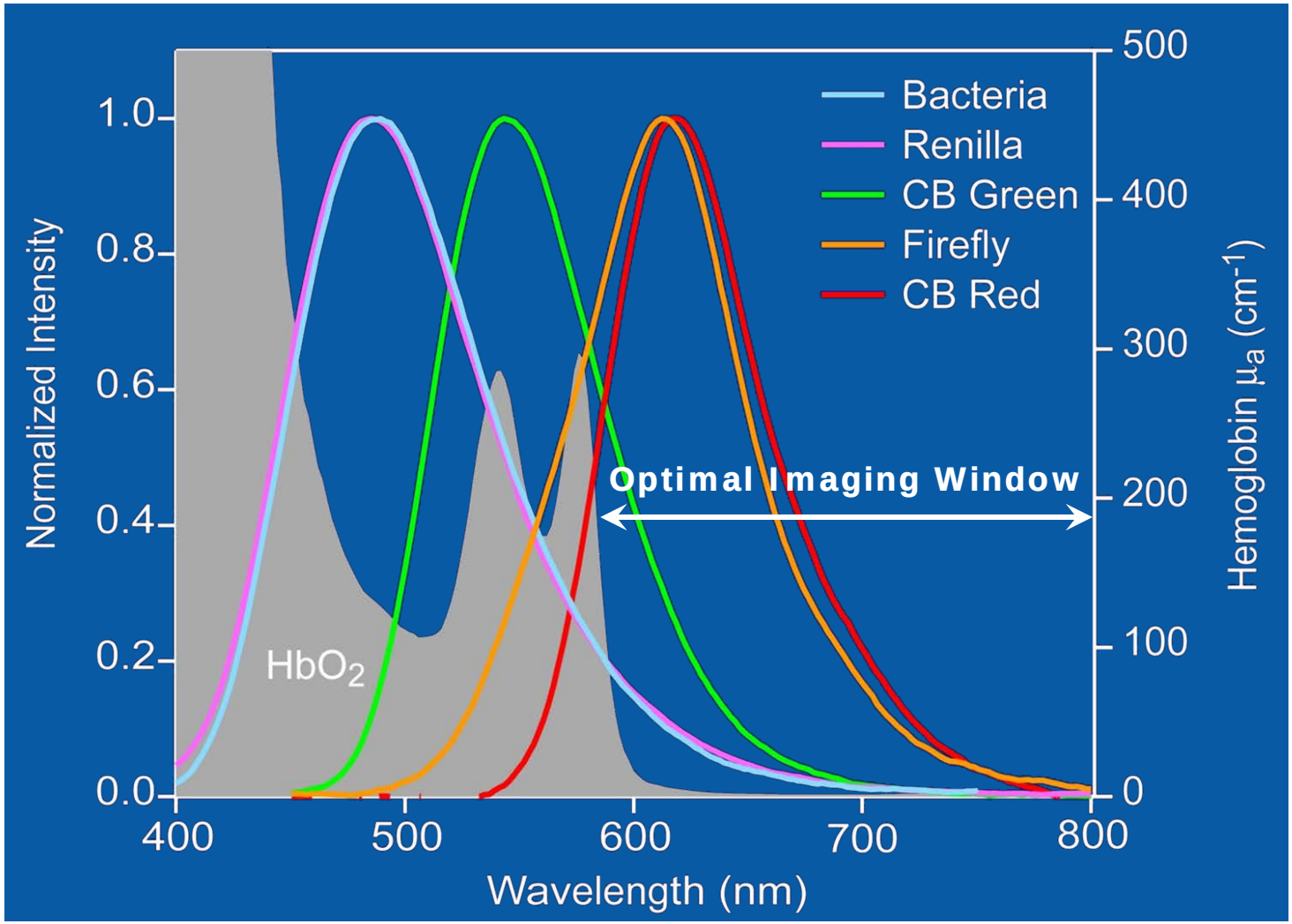


Spectral Unmixing: Extracts fluorescent signal from autofluorescence

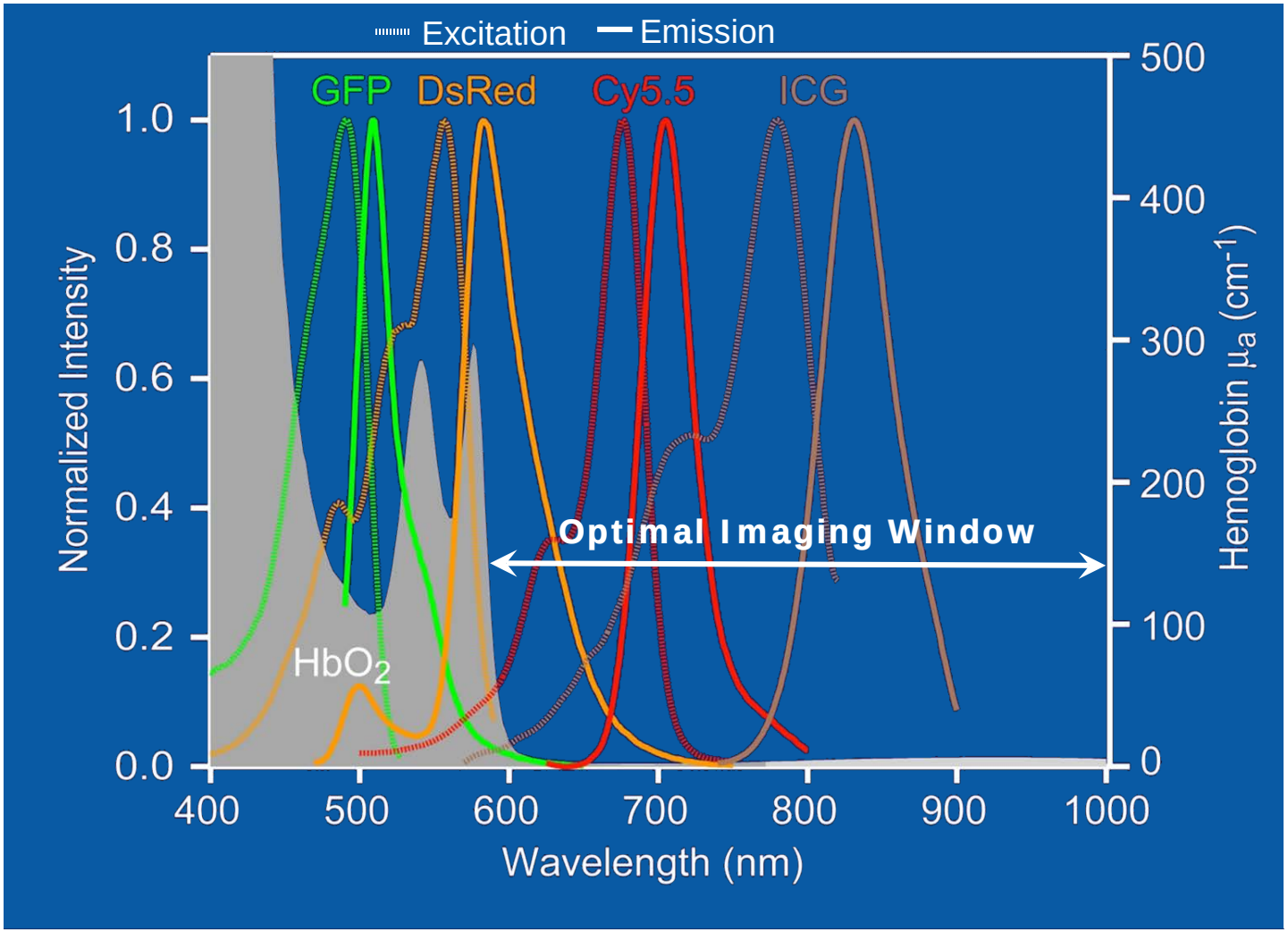




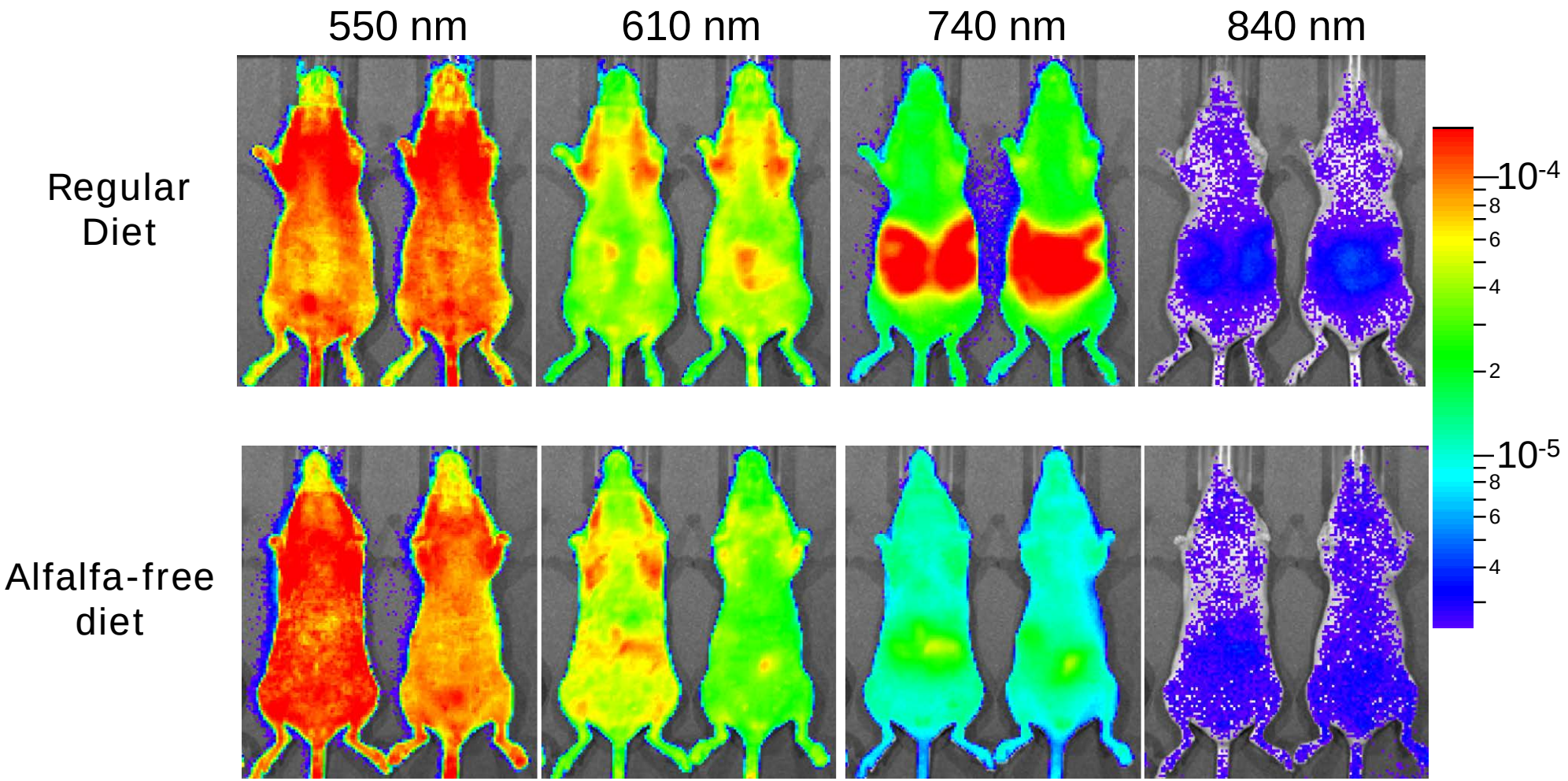
Emission Spectra of Common Luciferases



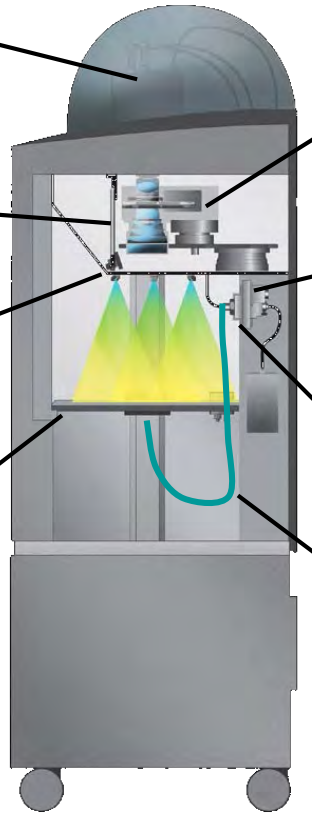
Emission Spectra of Common Fluorophores



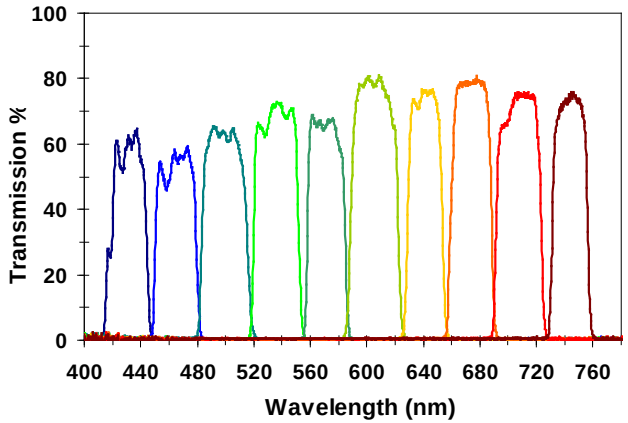
Autofluorescence Images of Control Mice



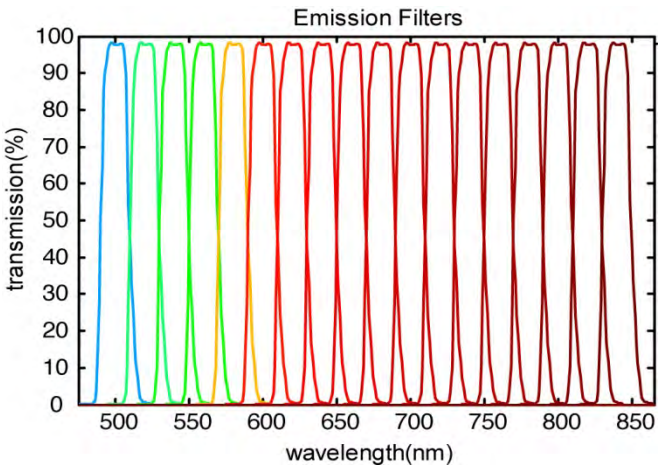
IVIS Spectrum



10 excitation filters



18 emission filters



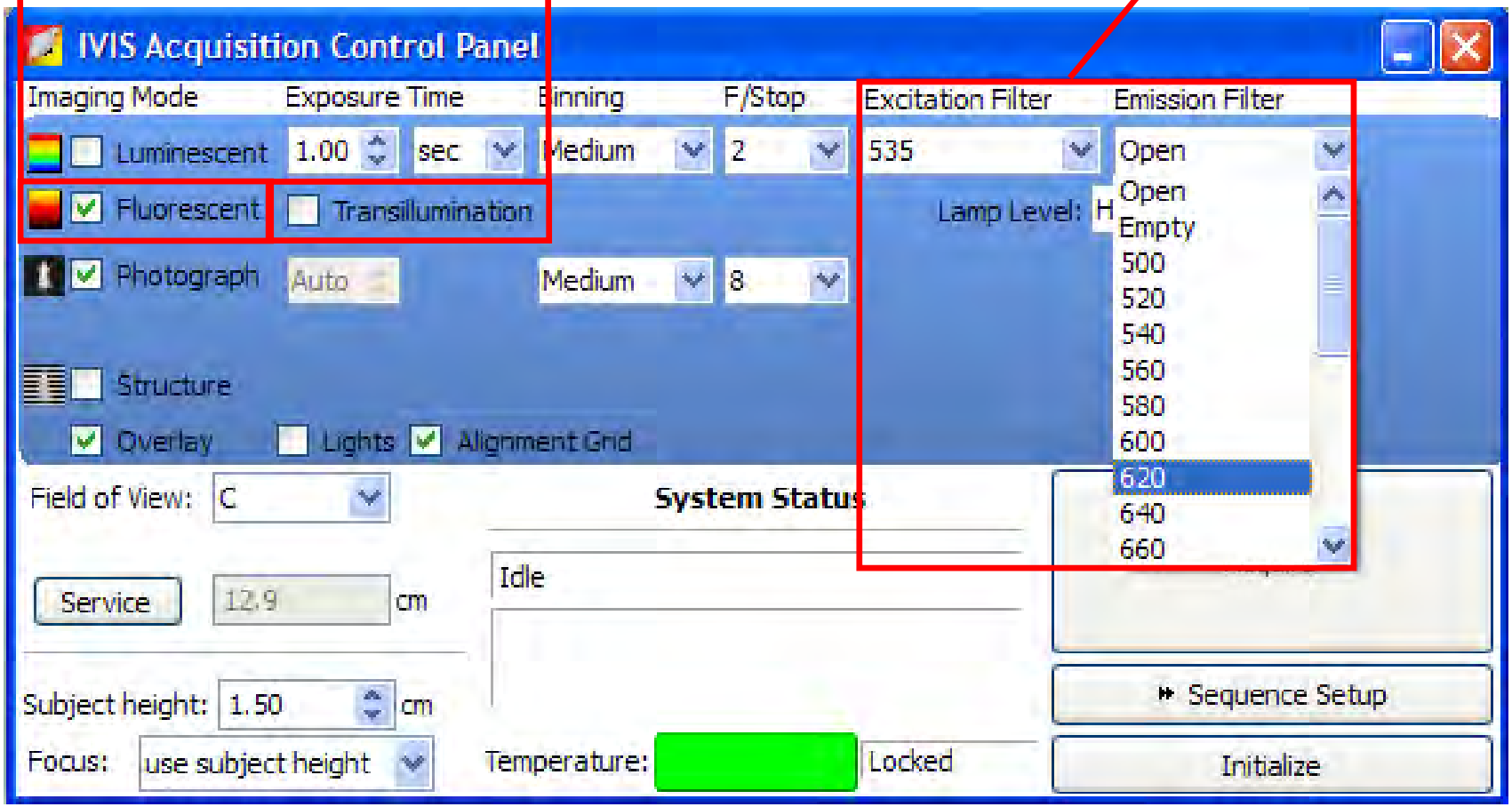
Fluorescence Acquisition

Select Fluorescent Imaging Mode

Trans-illumination

Lamp level High / Low

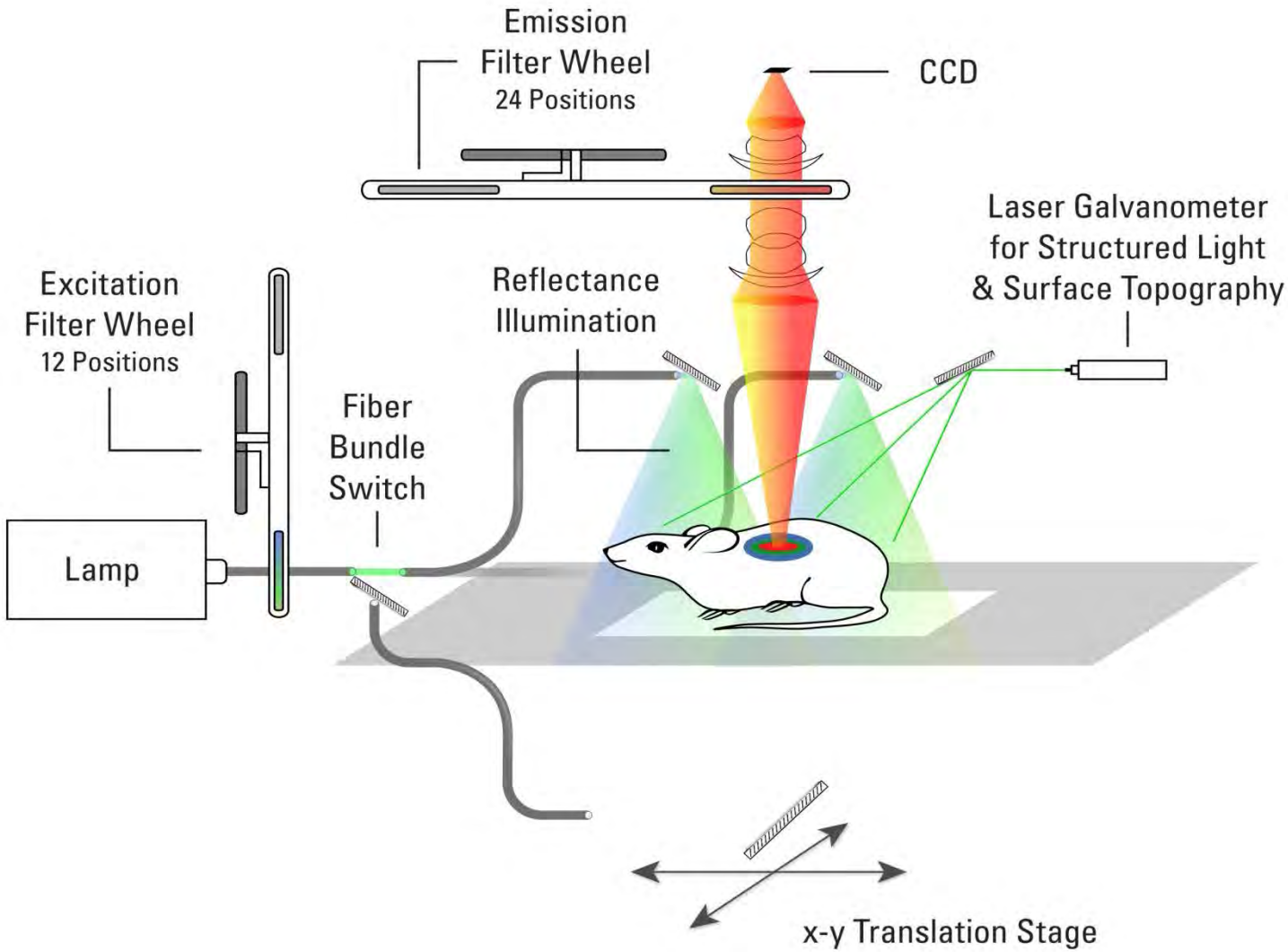
Select filters



Fluorescence

IVIS Spectrum Epi-illumination

Reflection-Mode (Epi) Illumination



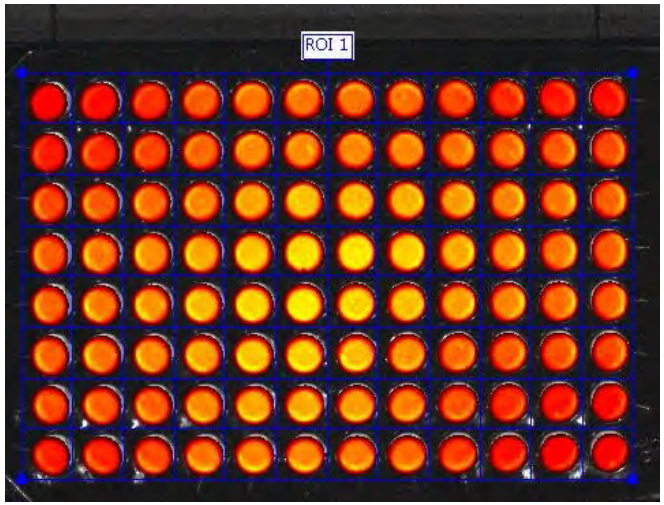
Fluorescent Calibrated Units: Radiant Efficiency

Excitation Light Pattern

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

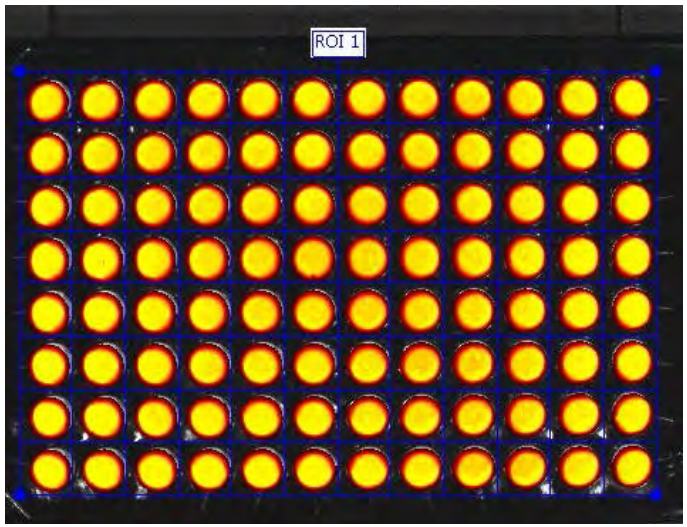
GFP Well Plate Uncorrected

Counts



vs.

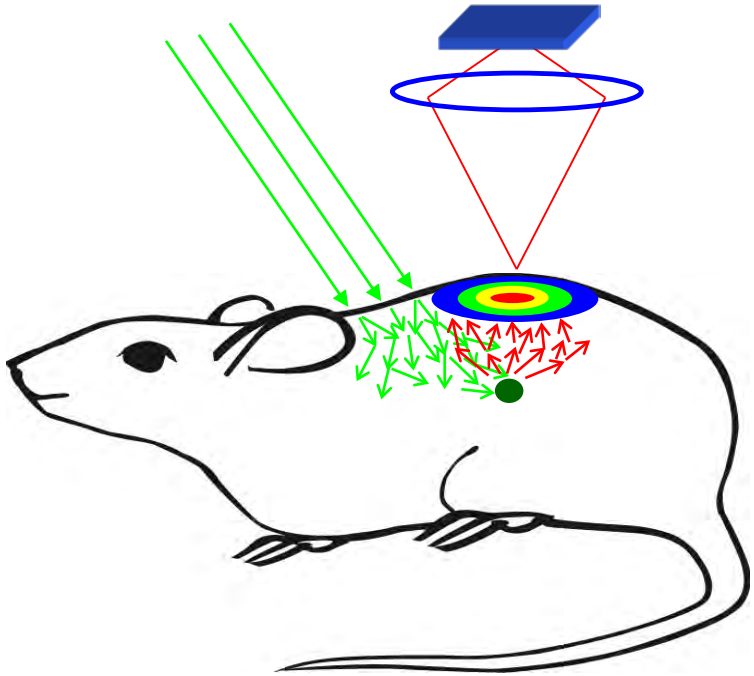
GFP Well Plate Corrected



Radiant Efficiency

Fluorescent Calibrated Units: Radiant Efficiency

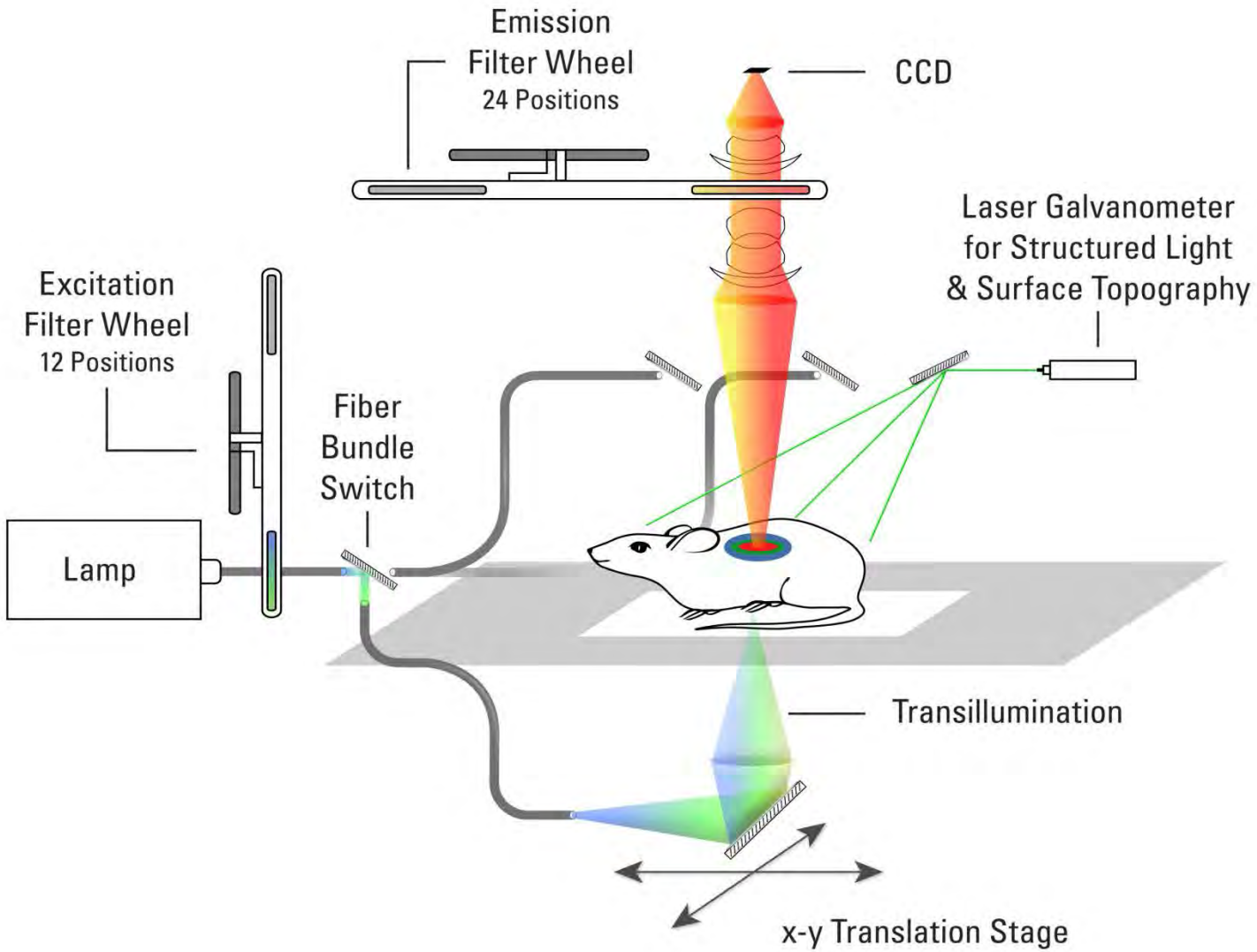
$$\text{Radiant Efficiency} = \frac{\text{Emission Light (photons/sec/cm}^2\text{/str)}}{\text{Excitation Light } (\mu\text{W/cm}^2)}$$



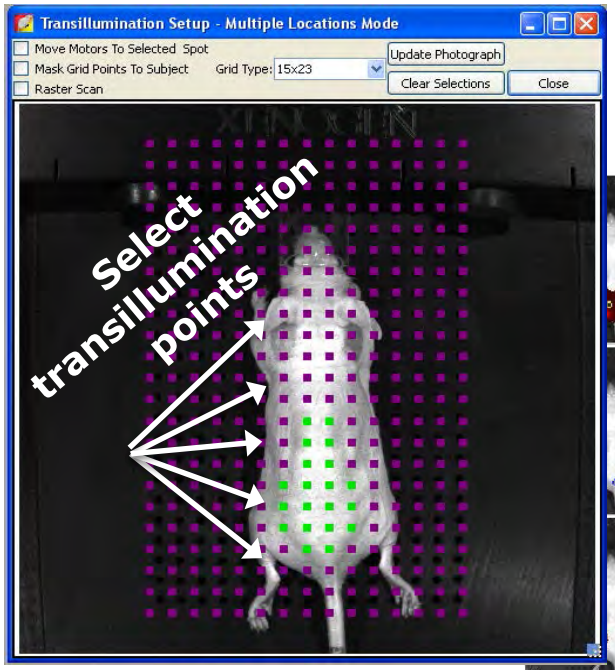
Fluorescence

IVIS Spectrum Transillumination

Transmission-Mode Illumination

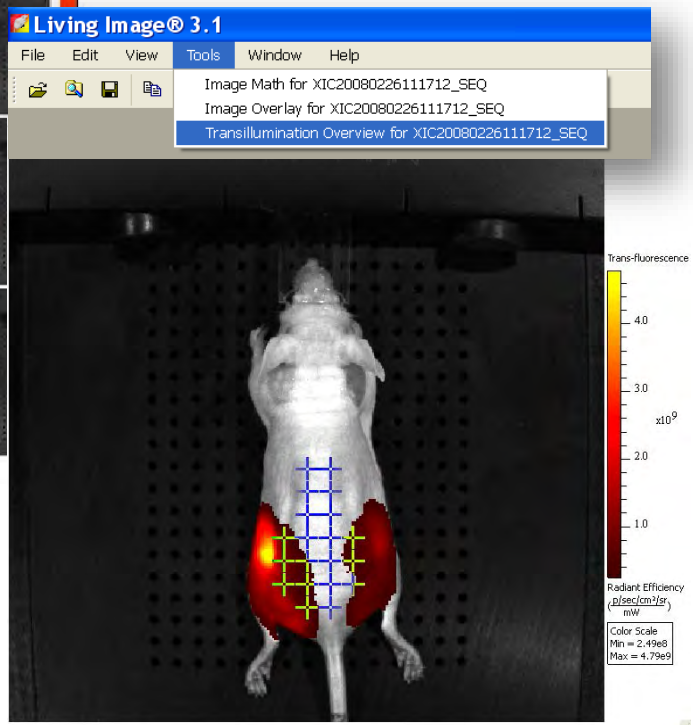


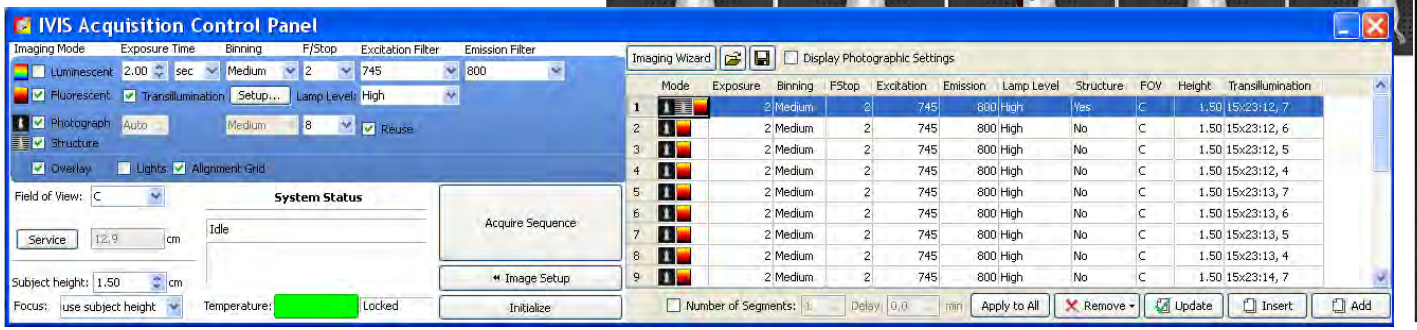
Transillumination Sequence Acquisition



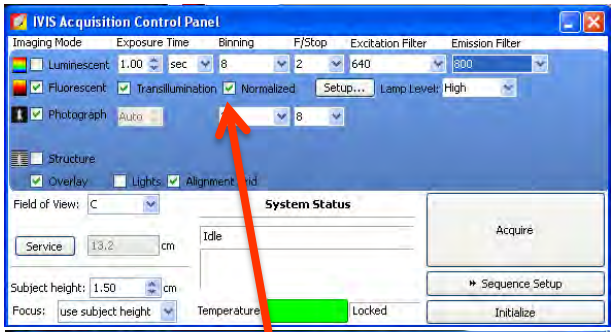
Single image taken for each point

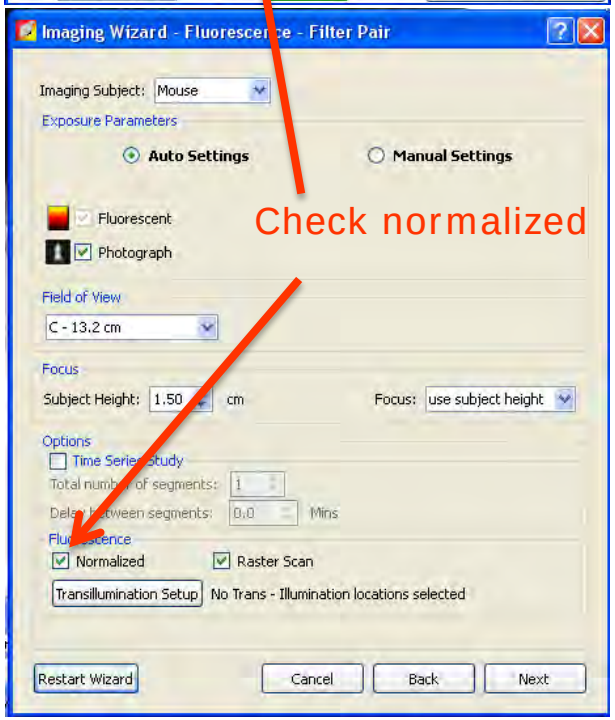
Overview combines points into one image



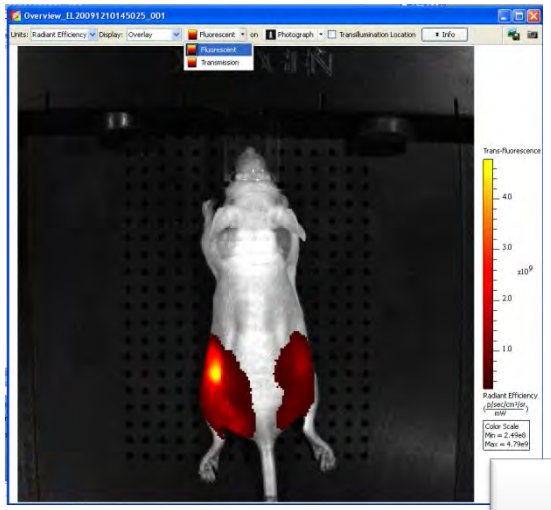


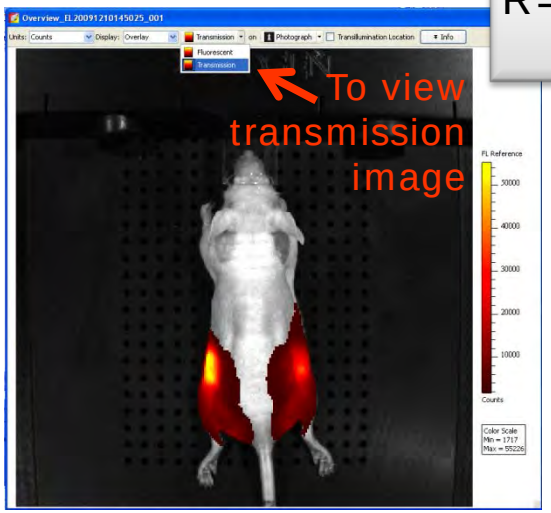
Normalized Transmission Fluorescence





Check normalized

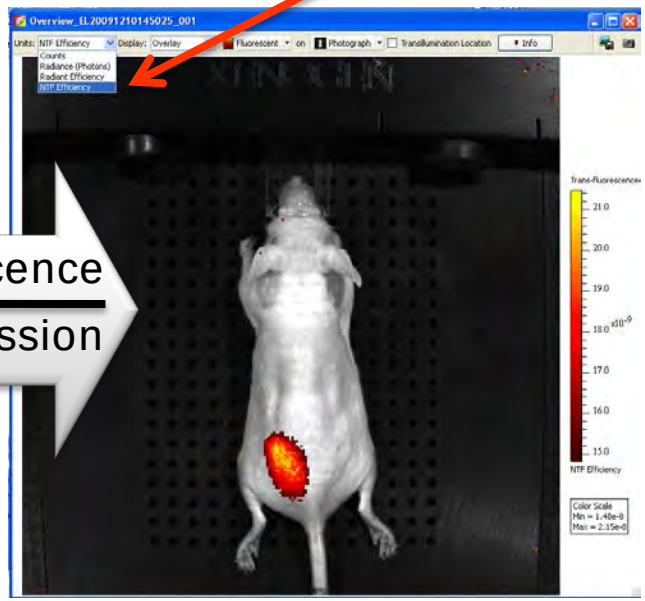




To view transmission image

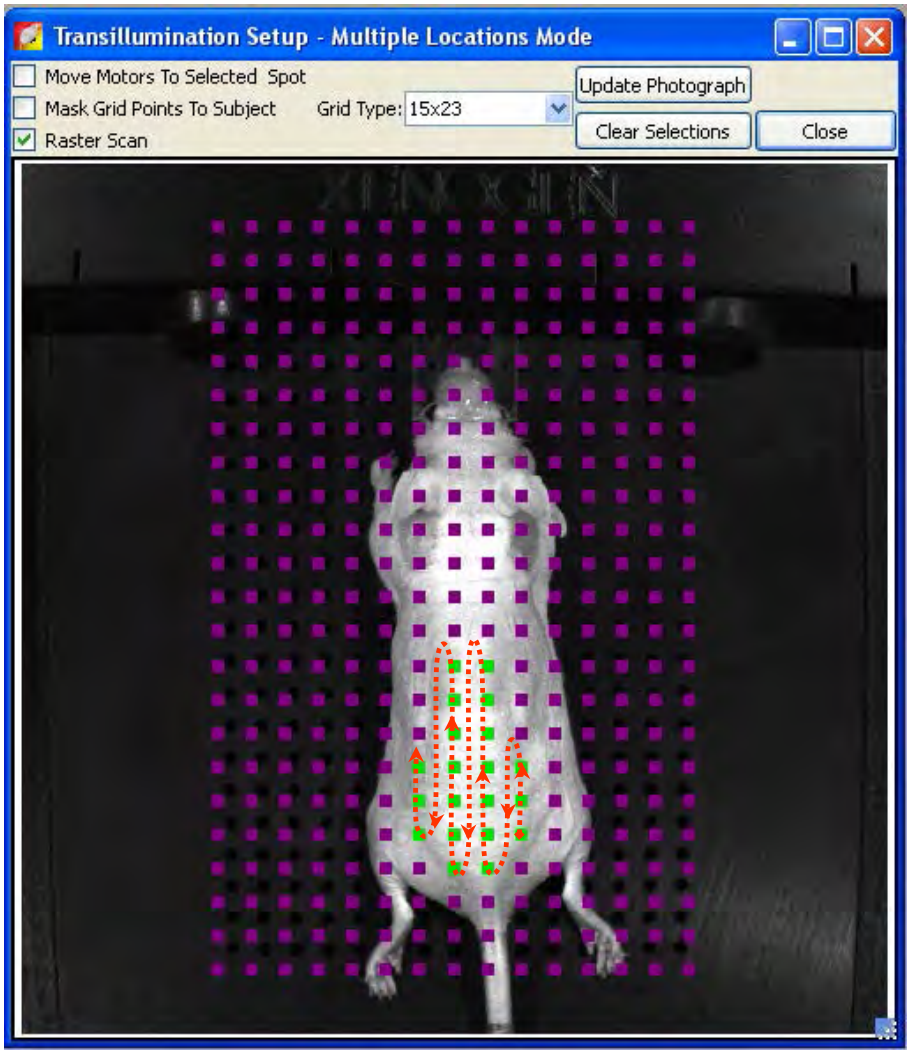
$$R = \frac{\text{Fluorescence}}{\text{Transmission}}$$

View normalized image



- Transmission measured at emission wavelength
- ND2 – neutral density filter

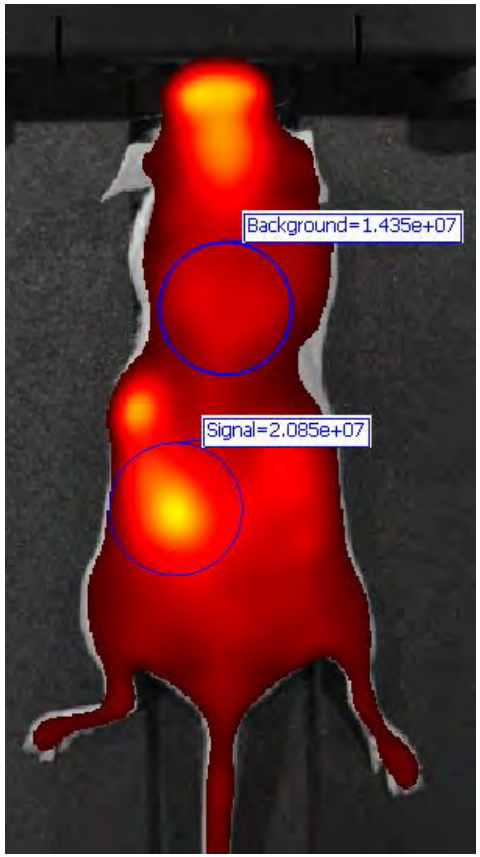
Raster Scanning Capabilities



- Faster
  - ➢ Shutter remains open as exciter moves from point to point
- Result is one image
- Can not be utilized for FLIT analysis

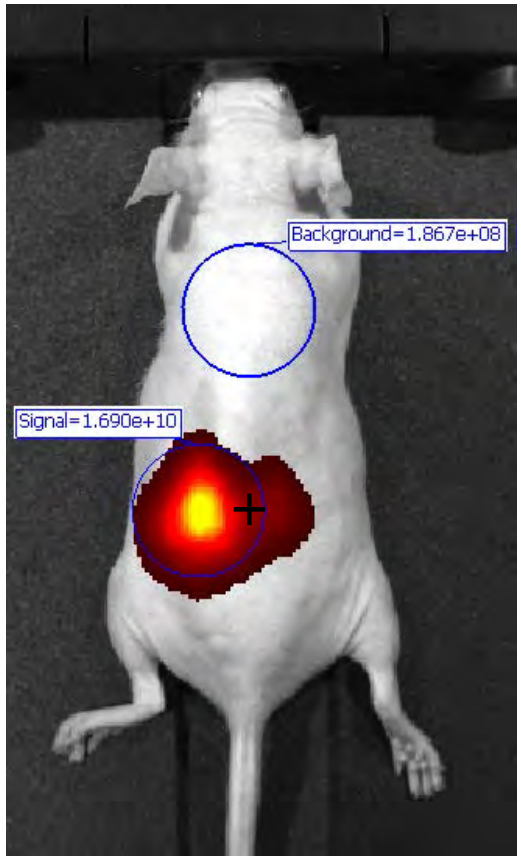
Transillumination Optimal for Deep Tissue Sources

Epi Illumination



sig/ bkg=1.43

Transillumination



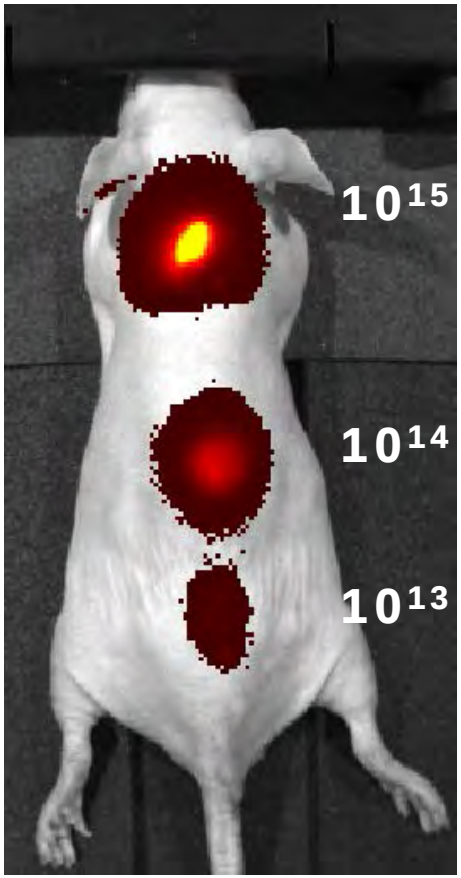
sig/ bkg=90.51

Ex 640 nm Em 700 nm

- XenoFluor 680: Pillow implanted medial to left kidney, $1x10^{15}$ molecules
- Intense source allows for more efficient excitation
- Autofluorescence lower
- Optical properties of reporter determine depth penetration

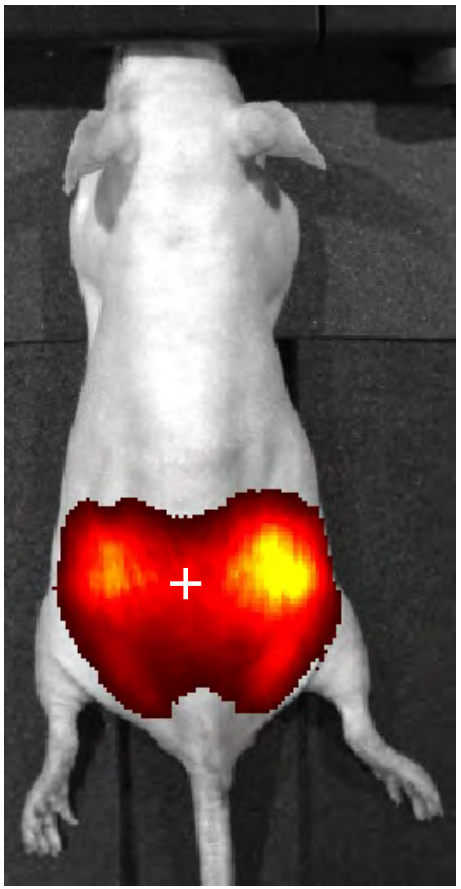
Reflection-Mode Imaging Reveals Shallow Signals Better than Transillumination

Epi Illumination



Ex 640 nm

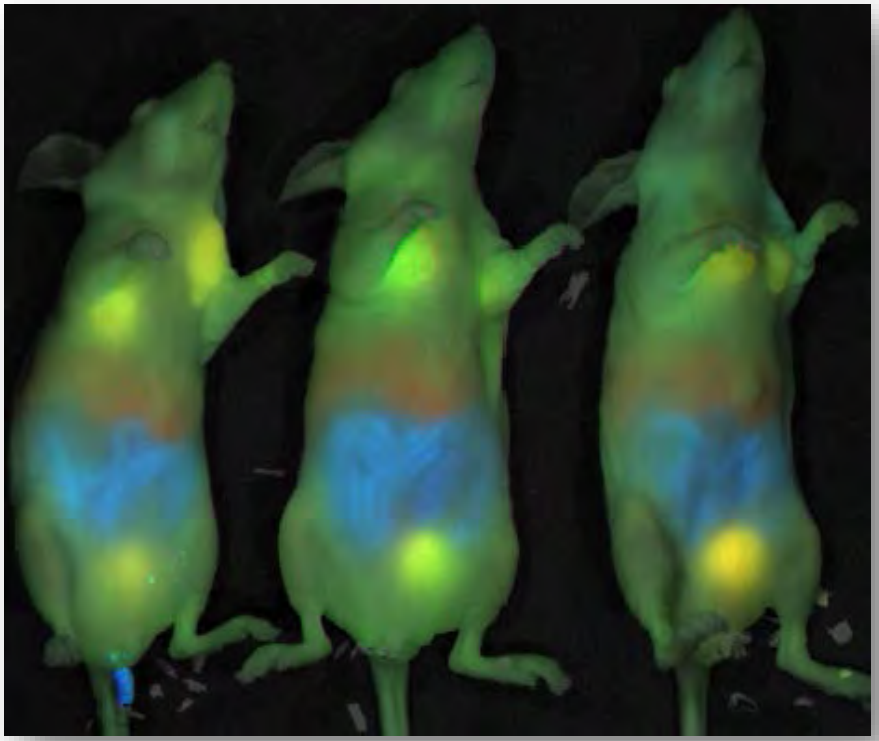
Transillumination



Em 700 nm

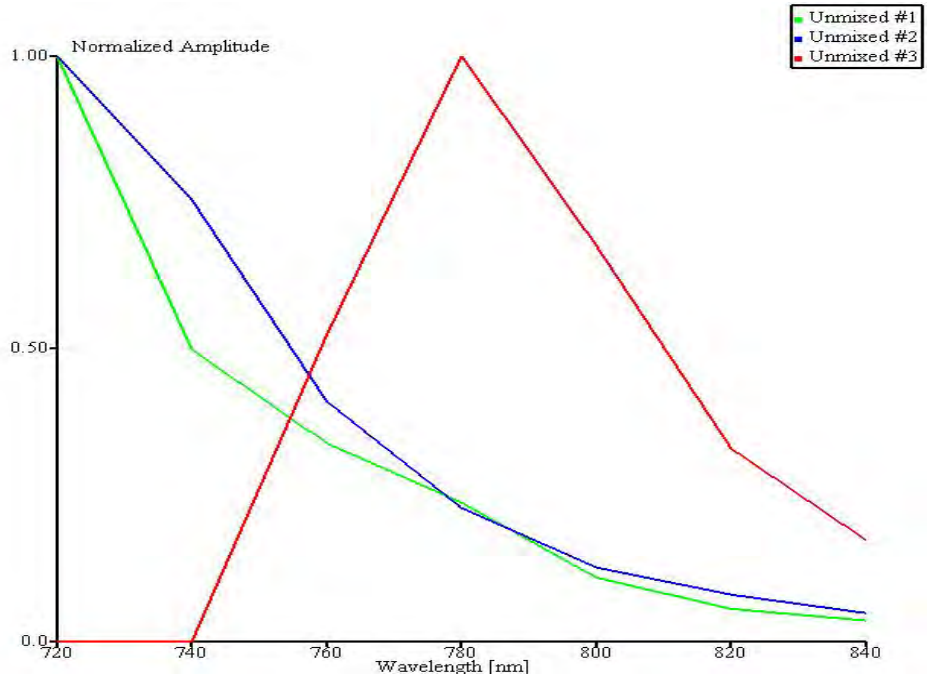
- XenoFluor 680: Subcutaneously injected - number of dye molecules shown
- Optical properties of reporter determine detectability at depth
  - Limits of detectability around 8mm with optimal reporter
- High throughput

What is Spectral Unmixing?

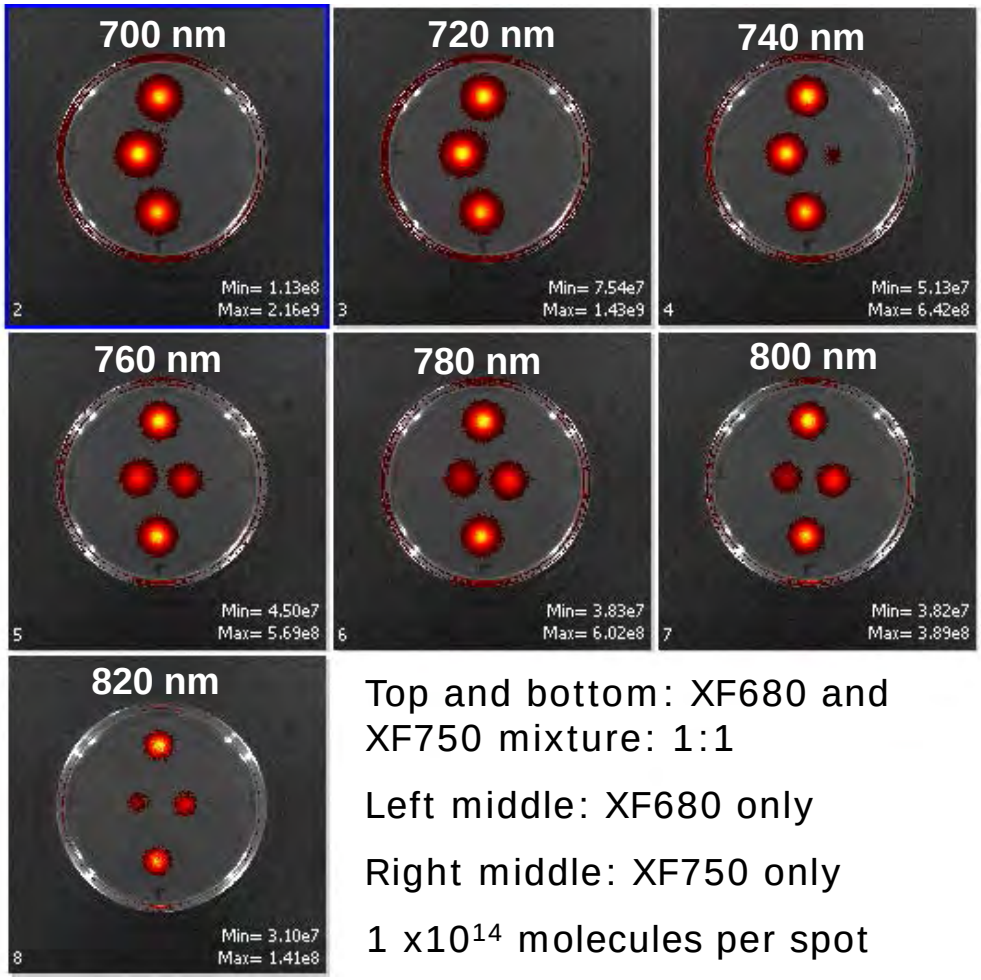


- Emission or excitation scan
- Quantitative and qualitative results

- Calculates concentrations of different fluorescent components
- Requires images acquired at multiple wavelengths to perform the spectral analysis



In vitro Spectral Unmixing Example:
Dyes in a Dish



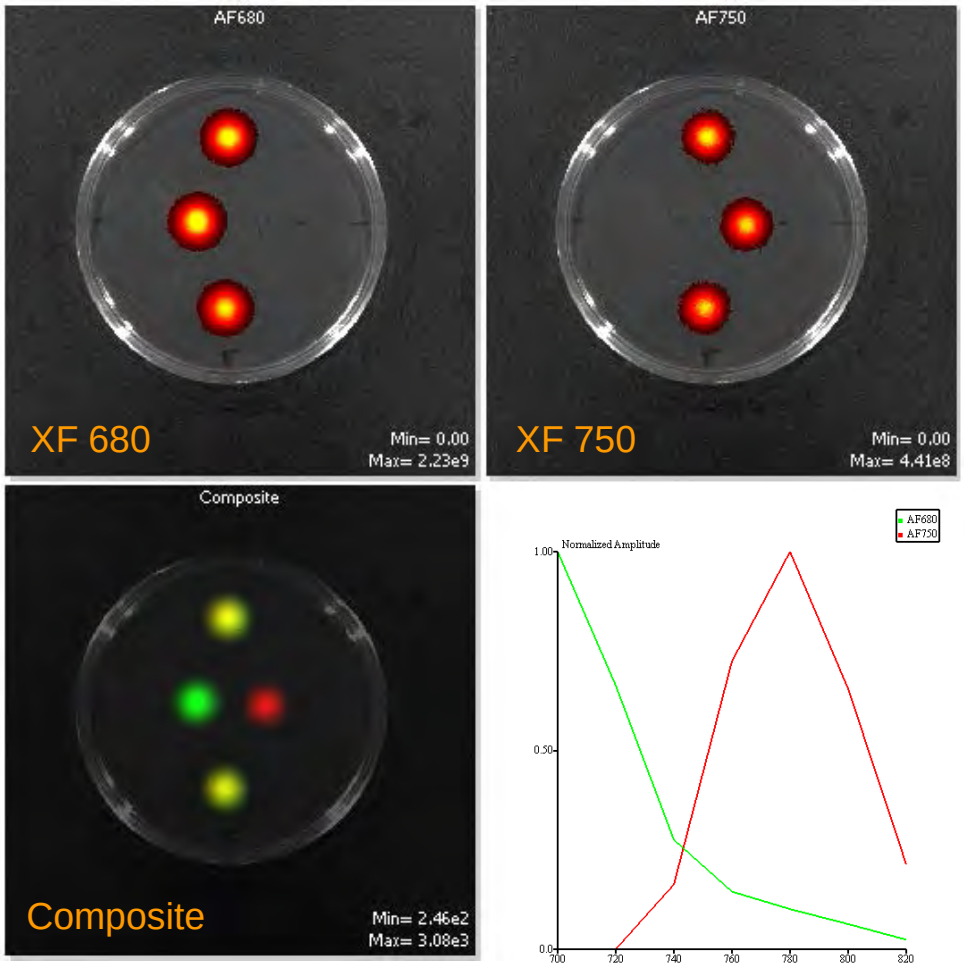
Top and bottom: XF680 and XF750 mixture: 1:1

Left middle: XF680 only

Right middle: XF750 only

1 x10^{14} molecules per spot

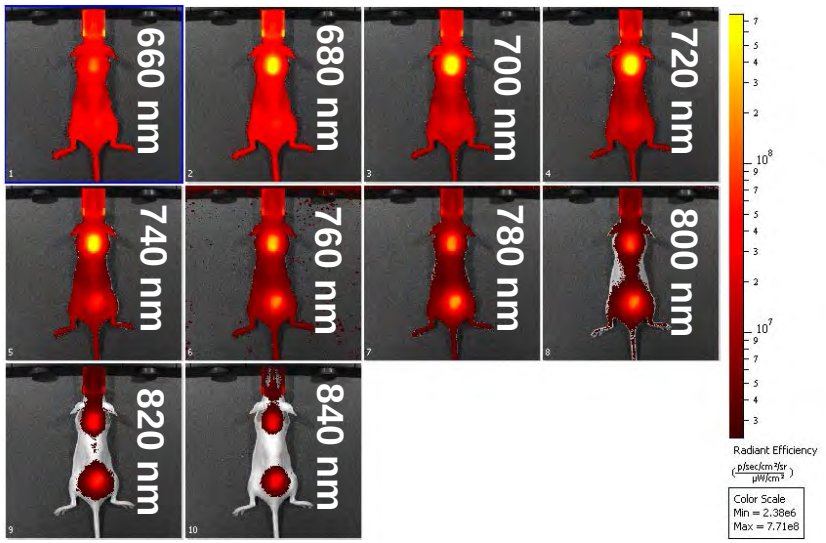
Ex640, Em700-820nm

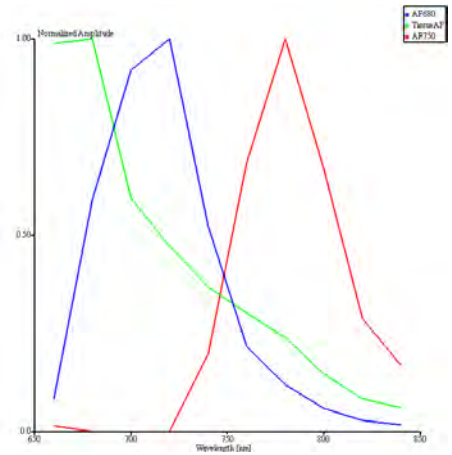


Spectral Unmixing (Epi-illumination)

XenoFluor 680/750

Raw Spectral Images

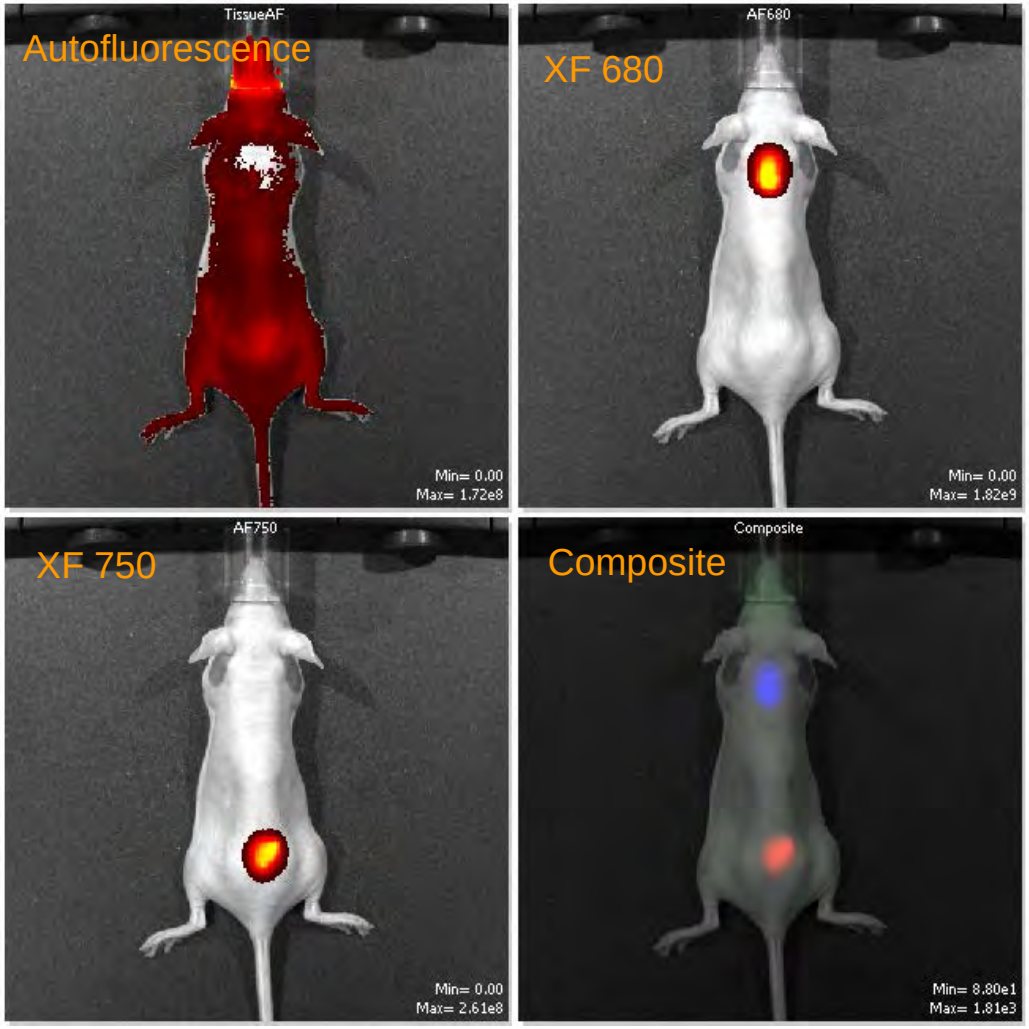




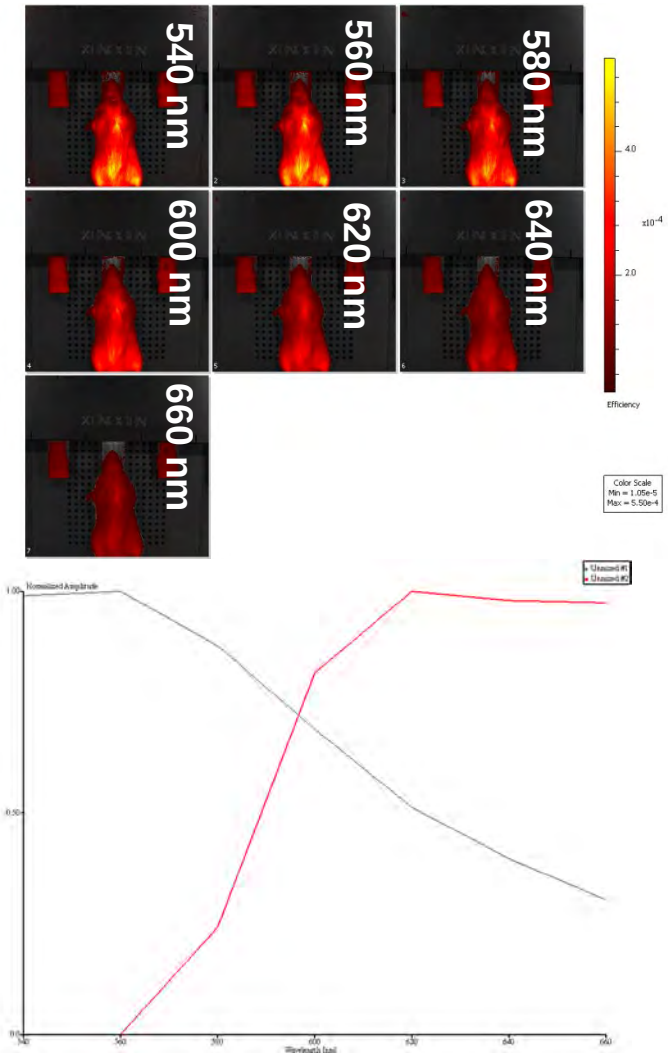
Subcutaneous injections of 10^{14} molecules of XenoFluor 680 (scruff)

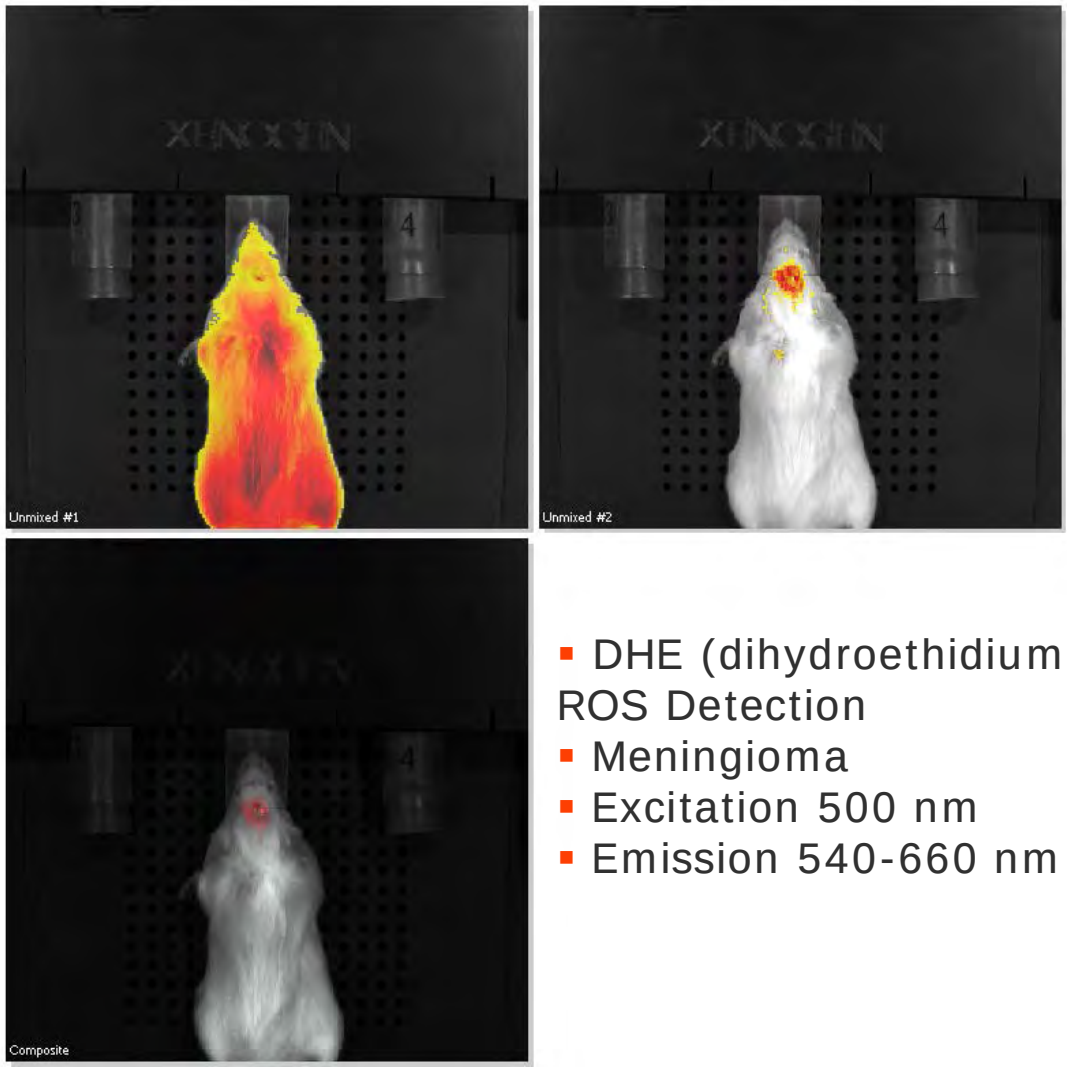
Subcutaneous injection of 10^{14} molecules of XenoFluor 750 (lower dorsal region)

605nm excitation filter



Spectral Unmixing of DHE in Brain

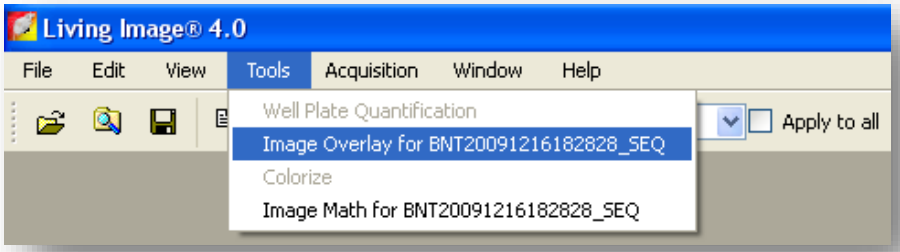


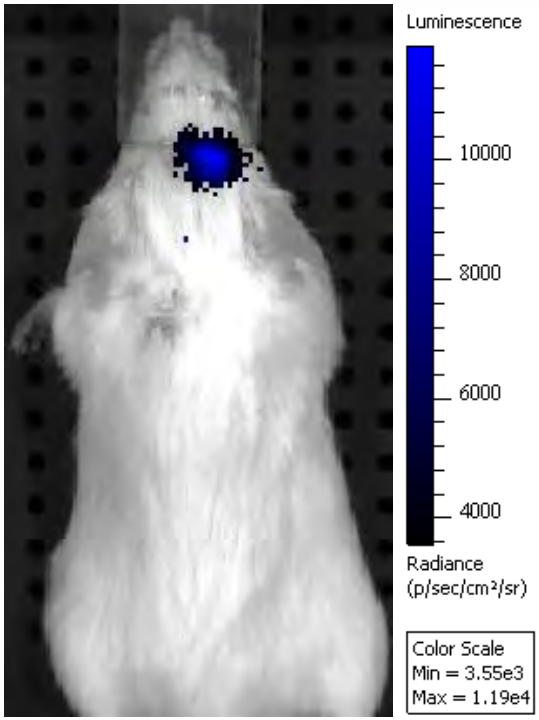


- DHE (dihydroethidium) ROS Detection
- Meningioma
- Excitation 500 nm
- Emission 540-660 nm

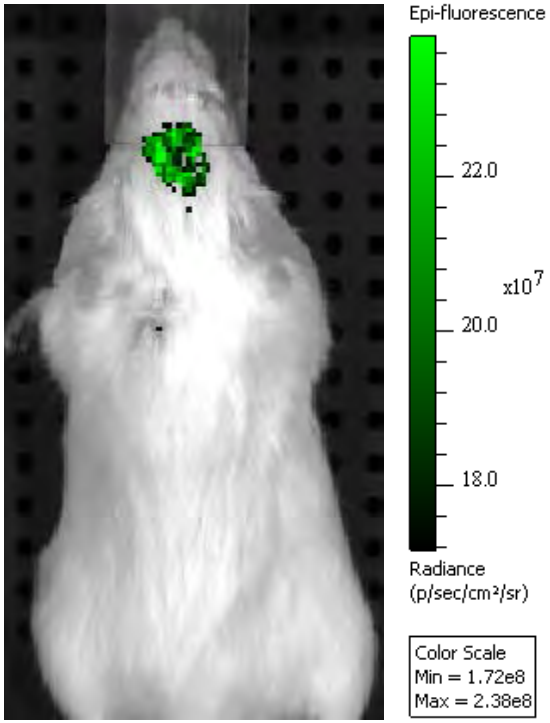
Data compliments of Adrienne Scheck St. Joseph's Research Hospital, Phoenix, AZ

Image Overlay Capabilities





Bioluminescent Tumor



DHE

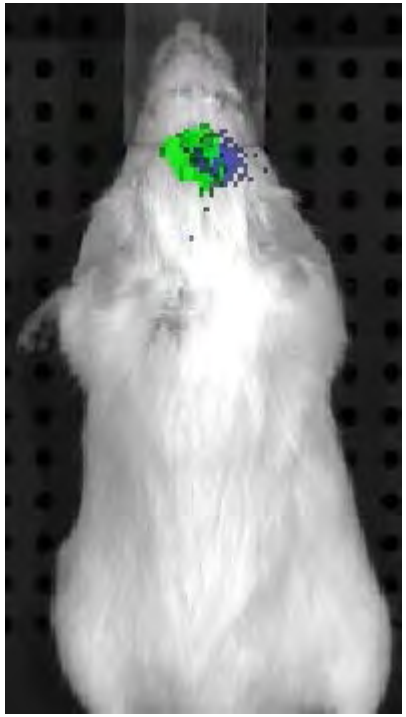


Image Overlay

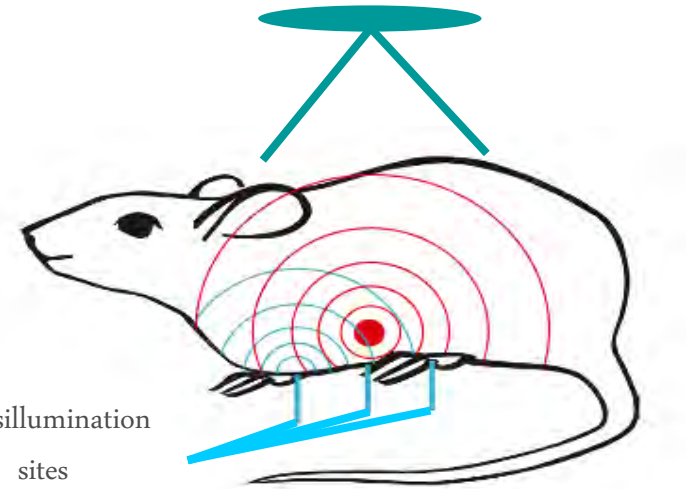
Single View 3D Imaging

Bioluminescence (DLIT™):

- Obtain top surface topography using structured light
- Use luminescent images at several emission wavelengths
- Solve for source location and brightness (flux)

Fluorescence (FLIT):

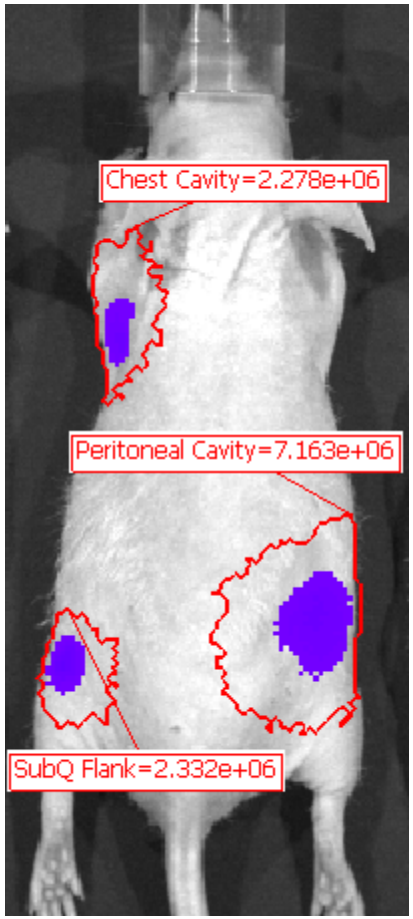
- Obtain top surface topography using structured light
- Use fluorescent images from multiple trans illumination scans



Transillumination sites

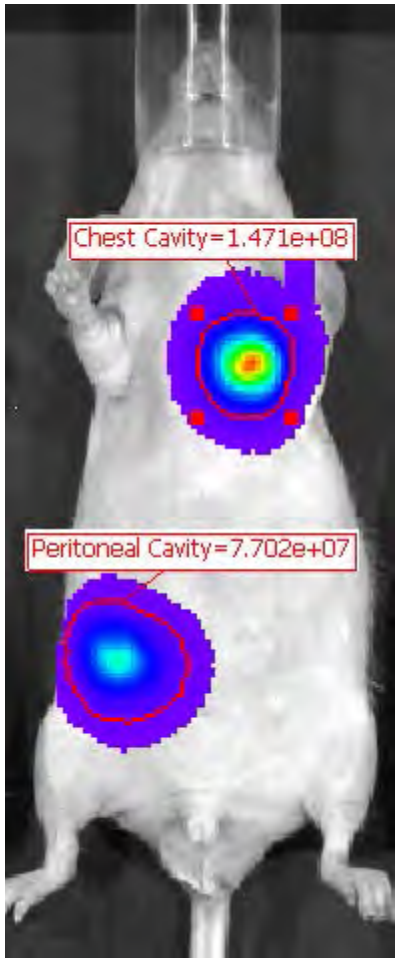
Do I need DLIT/FLIT?

Dorsal View



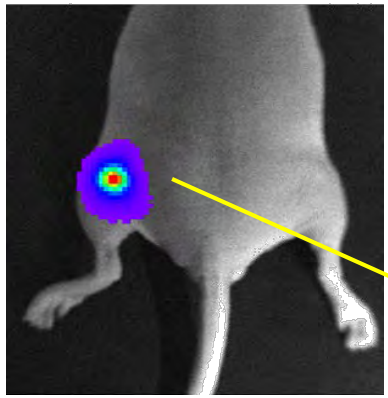
- Do you need to compare two foci directly?
- Determine the best view and stick with it
- Consistency is key

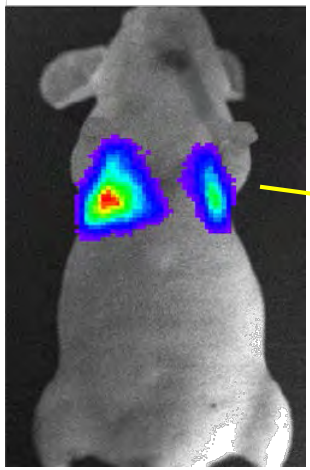
Ventral View

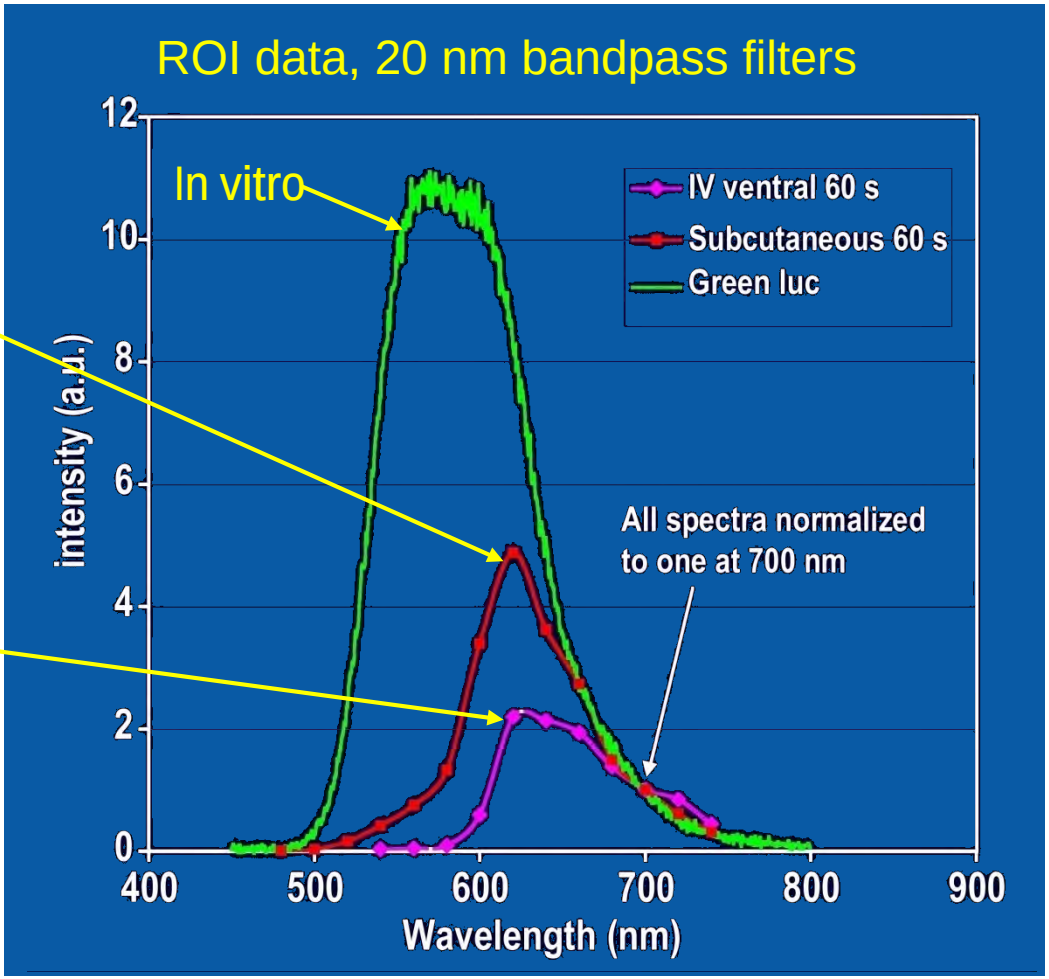


29 days after i.c. injection of $2x10^6$ PC3M cells

Spectral measurements provide information on depth of source



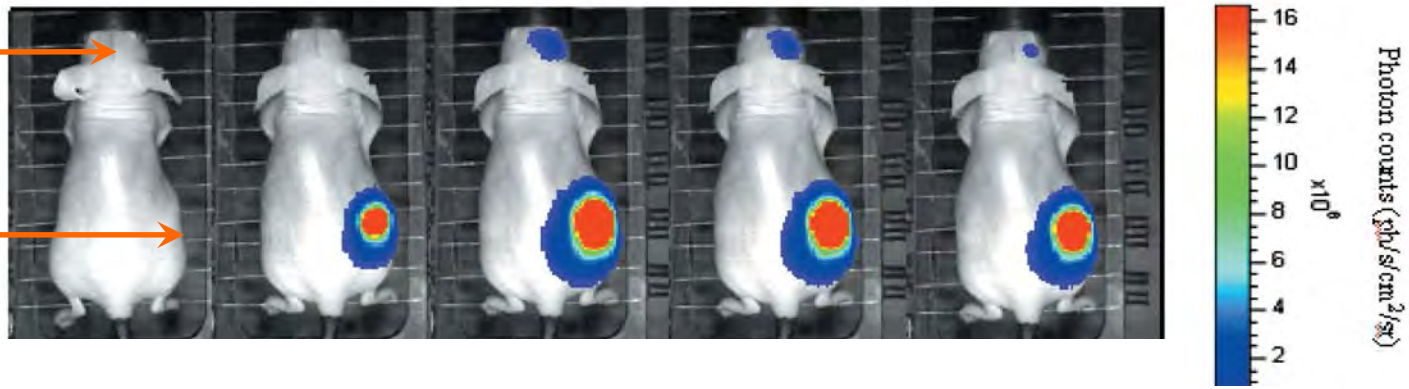


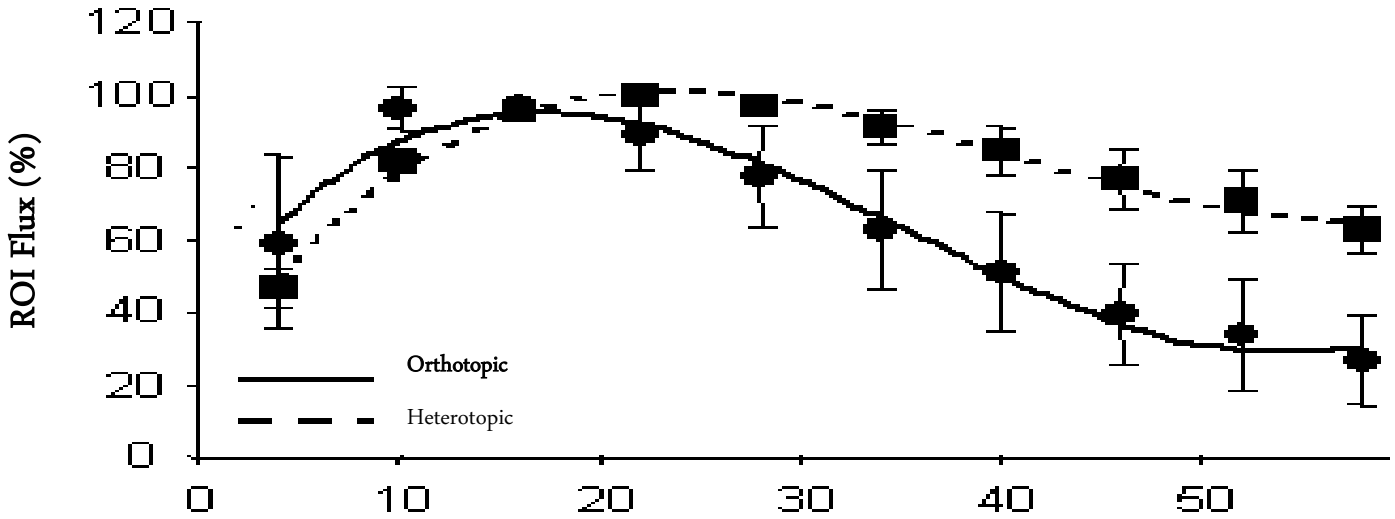


In-depth Knowledge of Luciferin Kinetics Essential for 3D Reconstruction

$1x10^5$ U87MG^{luc} cells in forebrain

$1x10^6$ U87MG^{luc} cells subQ

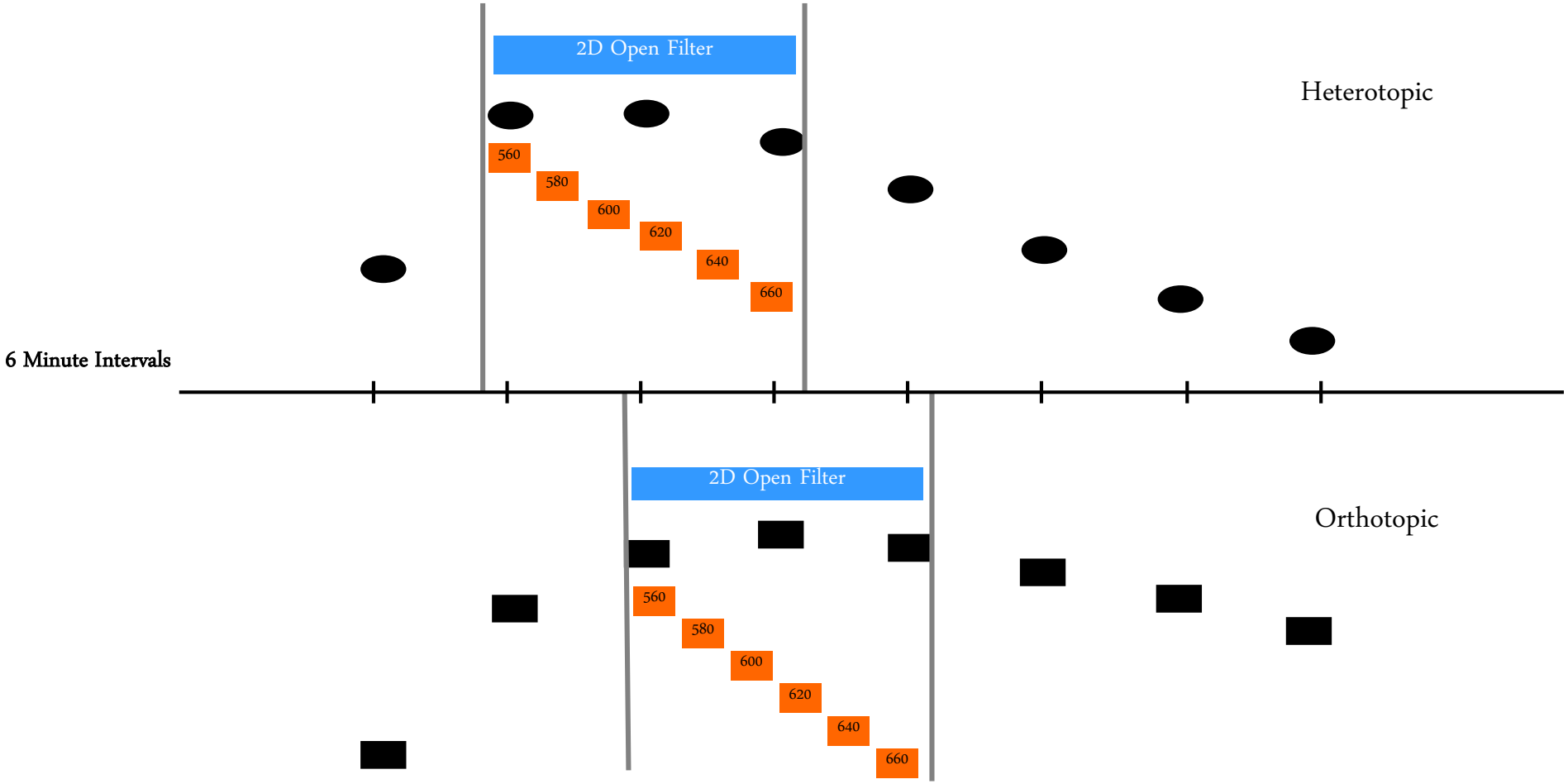




Burgos et al., 2003

3D Requires Multiple Spectral Measurements

Consistent light output assumed



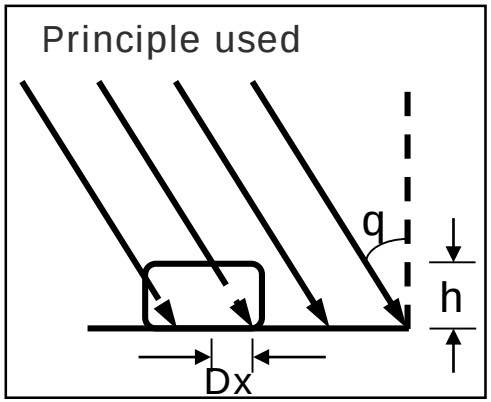
Burgos et al., 2003

Surface Topography / Diffuse Tomography (DLIT™)

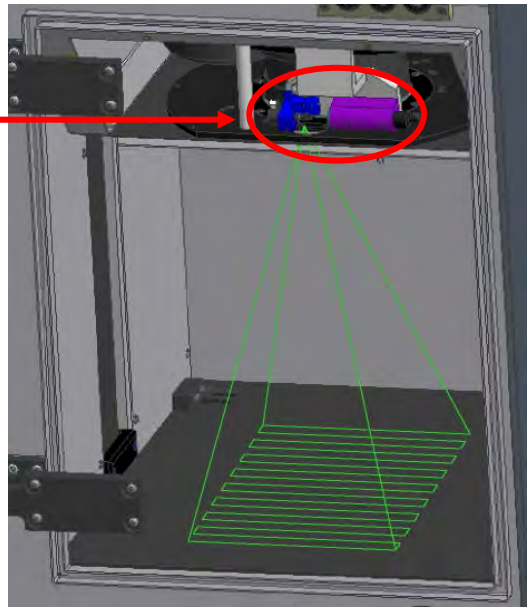
- Acquire a sequence of photographic and bioluminescent images at multiple wavelengths. Acquire one structured light image in sequence.
- Use structured light images to reconstruct surface mesh of mouse
- From surface radiance images, determine the photon density just inside the surface on every element of the surface mesh
- Divide the volume of the object into a grid of cubic voxels
- Define and solve a system of linear equations that relate the source strength of each voxel to the radiance at each surface element using diffusion theory with approximate boundary conditions
- Display resulting source strengths and locations

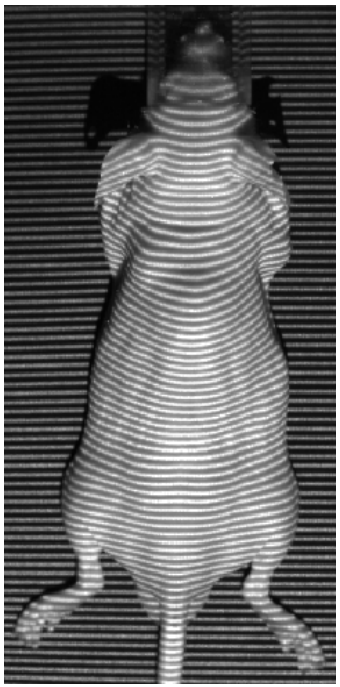
Surface Topography Reconstruction

Structured Light Image provides single-view surface topography (top surface)



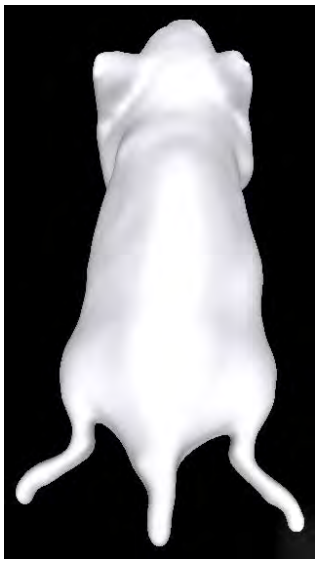
Structured
Light Projector



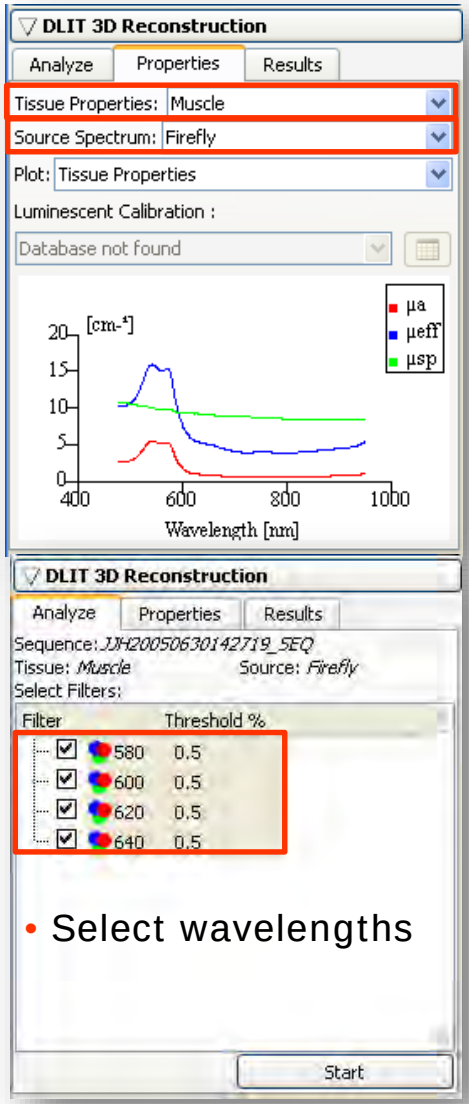


Structured
Light Image

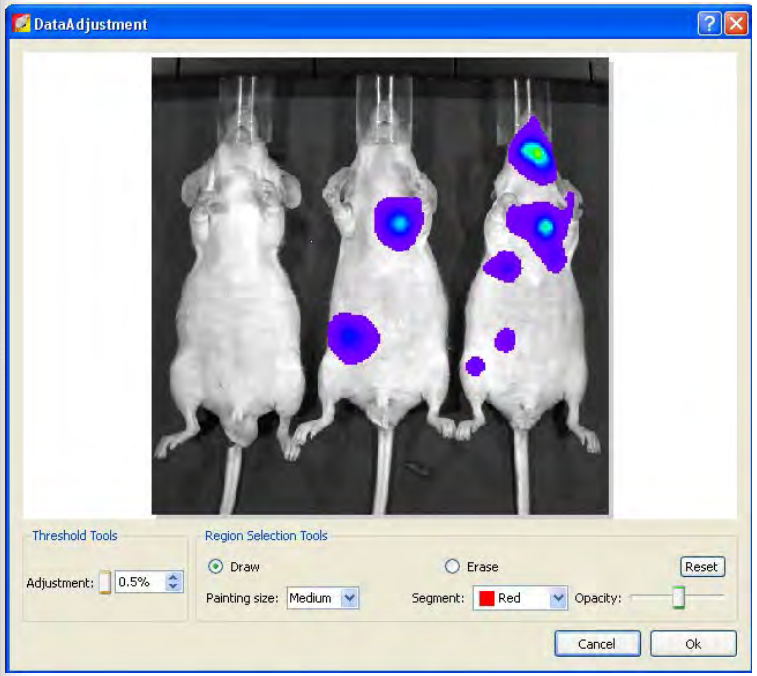
Height Map



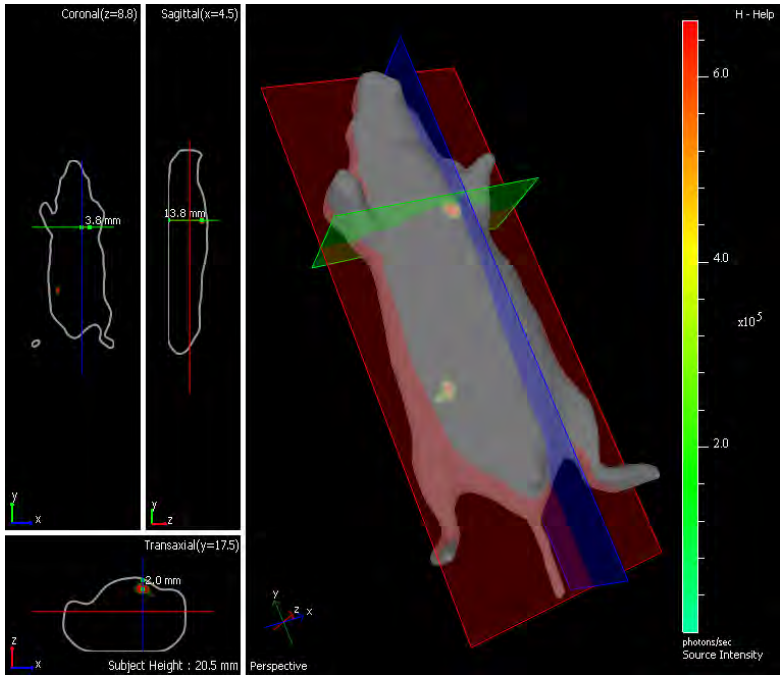
DLIT™ Reconstruction



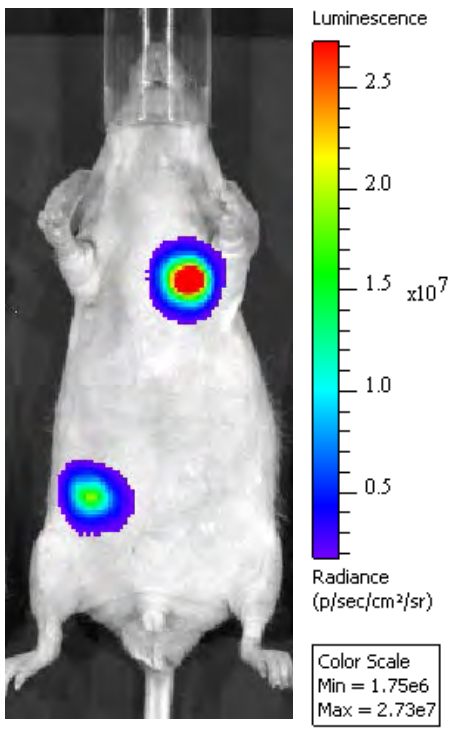
- Select tissue properties
- Select source spectrum
- Select wavelengths

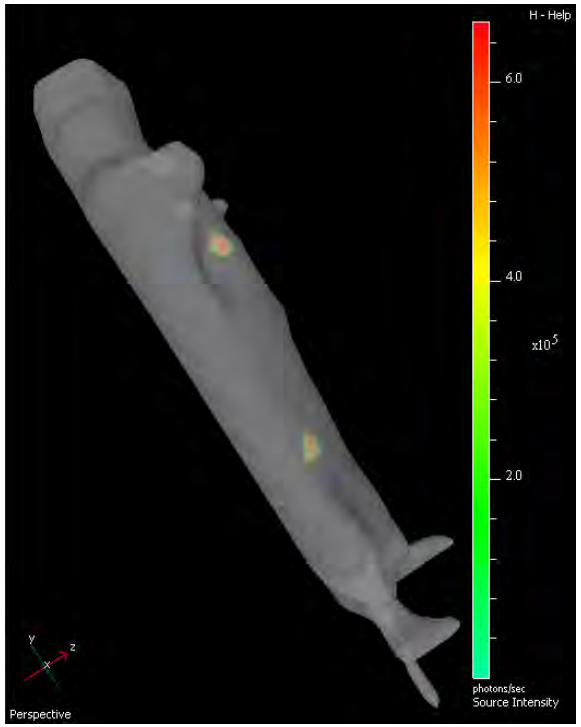


- Threshold your data



PC3M Intracardiac Metastatic Model





29 days after i.c. injection of 2×10^6 cells

λ = 580, 600, 620 nm

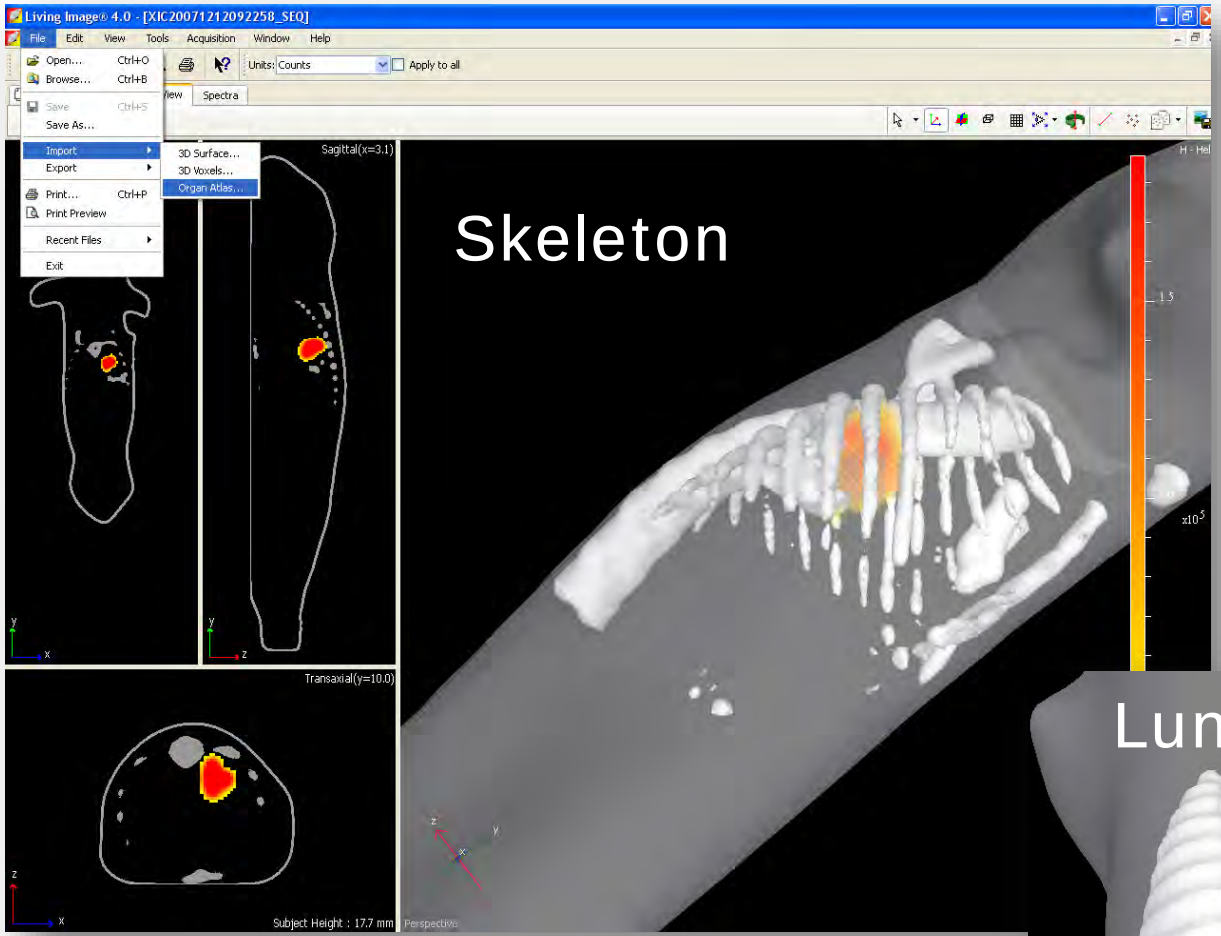
Chest Cavity		Peritoneal Cavity	
Depth [mm]	Flux [photons/sec]	Depth [mm]	Flux [photons/sec]
2.1	2.43×10^8	3.2	1.44×10^8

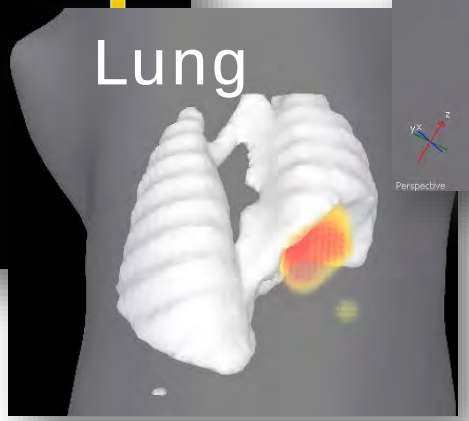
Automatic Mouse Atlas Registration in LI4.0

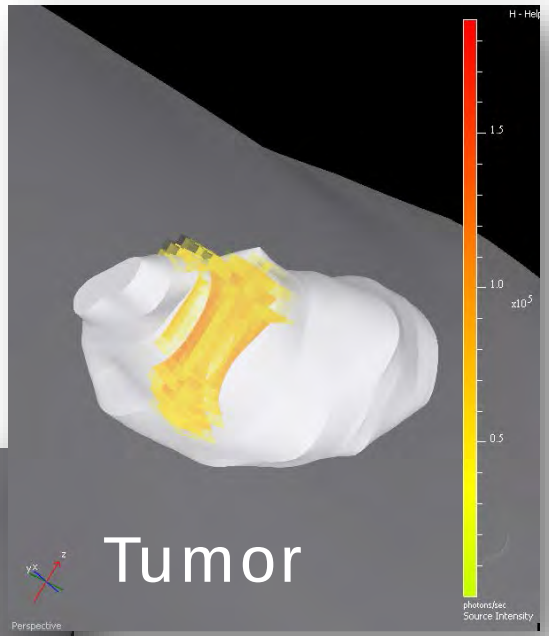
Unregistered

Coregistered

Coregistration with CT or MRI



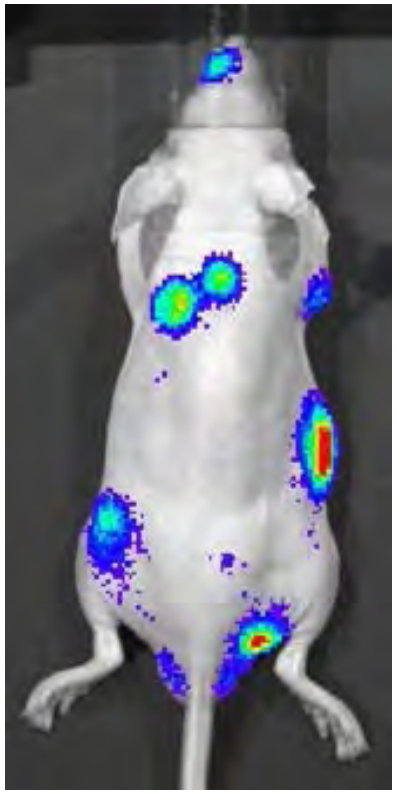


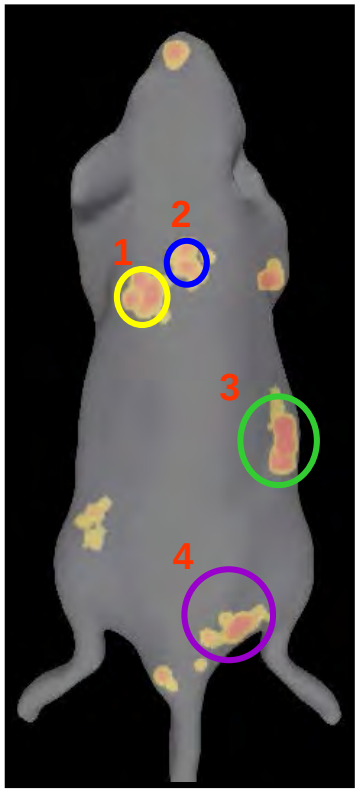


- Open Inventor (.iv) file format

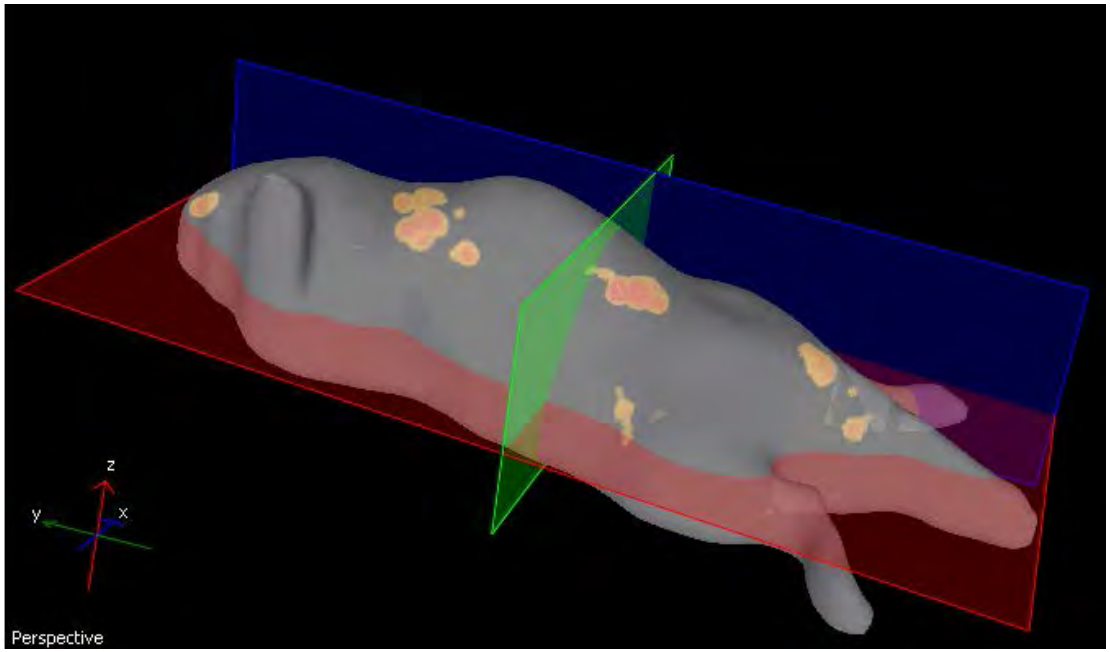
Multi-Wavelength 3D Reconstruction of B16F10 Melanoma Metastases Model

Dorsal View

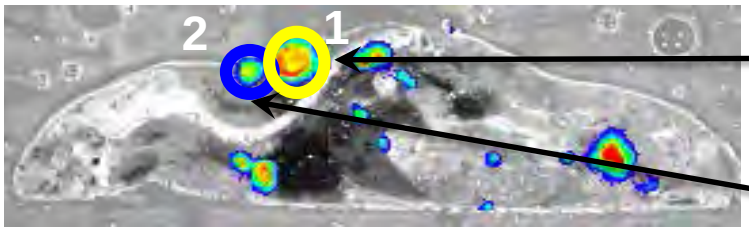




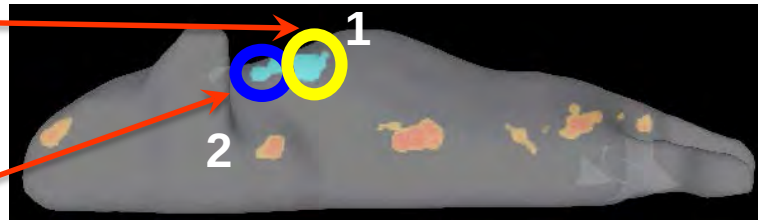
- 5x10^5 cells, injected IV
- Imaged on day 17
- Five filters from 560-640 nm

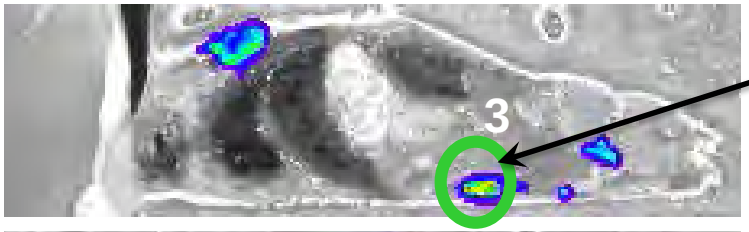


Tissue Section Analysis of Source Depth

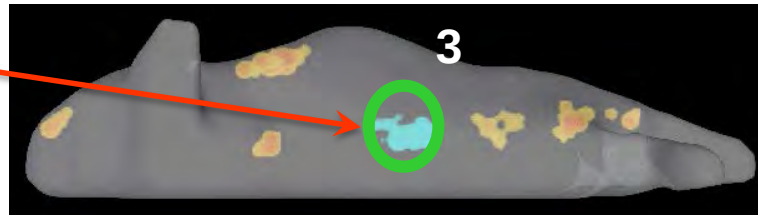


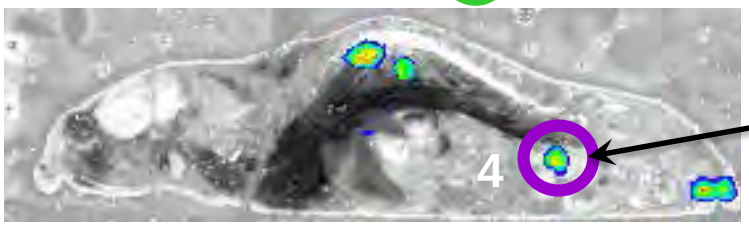
Actual (mm)	DLIT (mm)
3	2.5
2	1.5



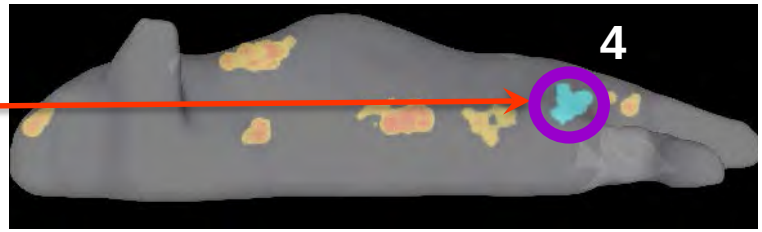


2 (from ventral surface)	8.2 (from ventral surface)



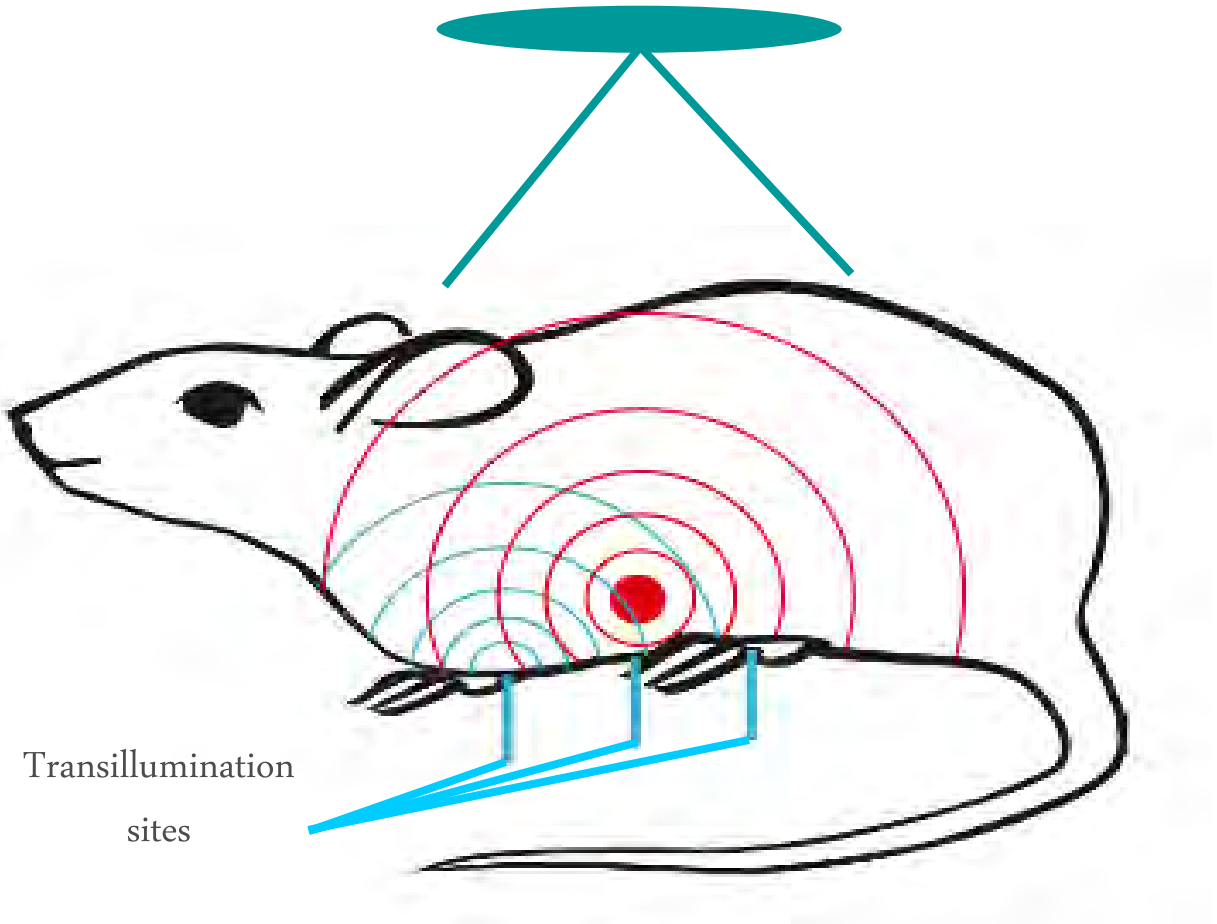


4	2.9



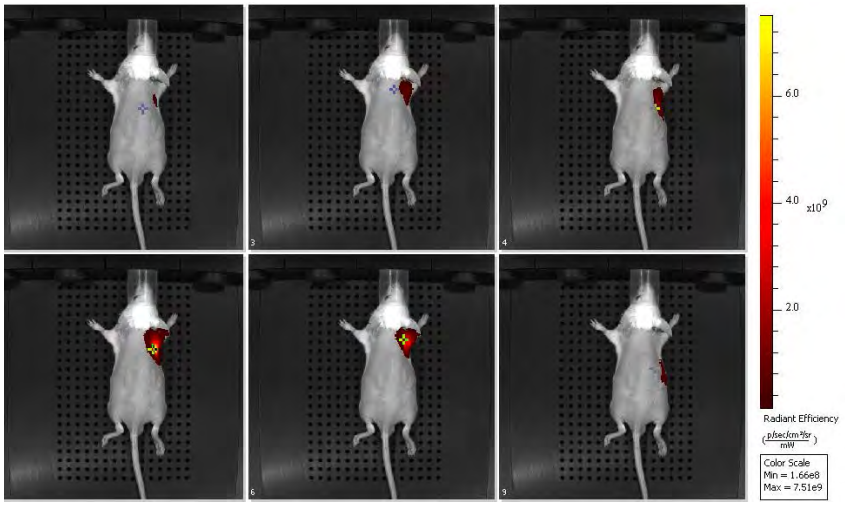
Determine best orientation – can reconstruct dorsal, ventral, left and right saggital

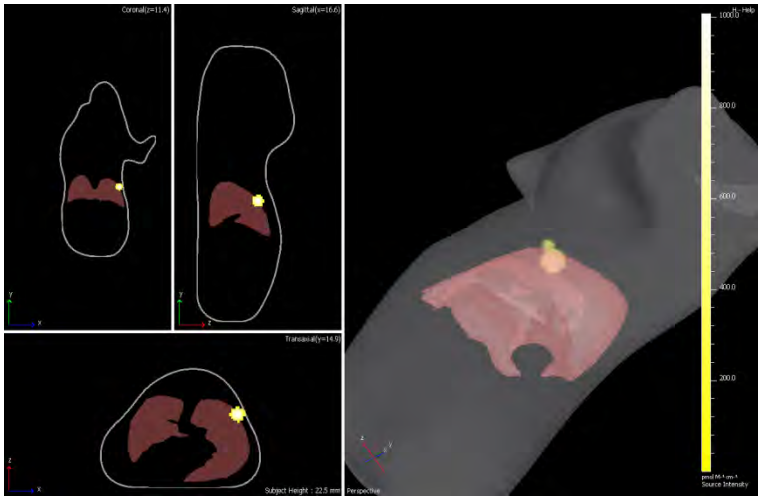
Transillumination Combined with FLIT can localize both shallow and deep tumors in 3D

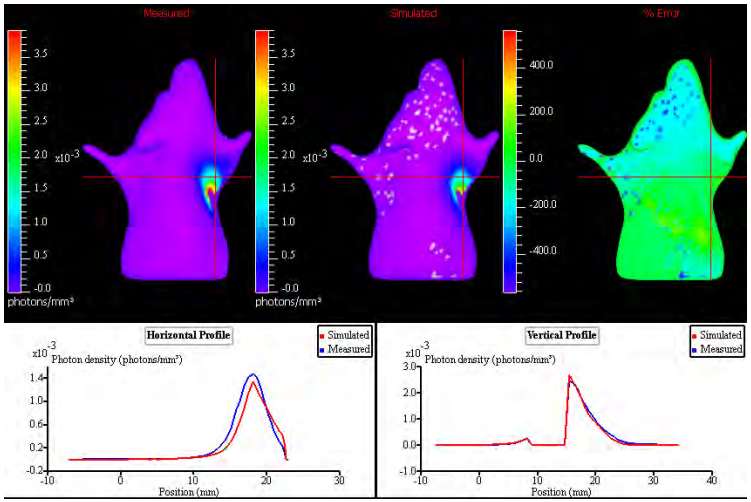


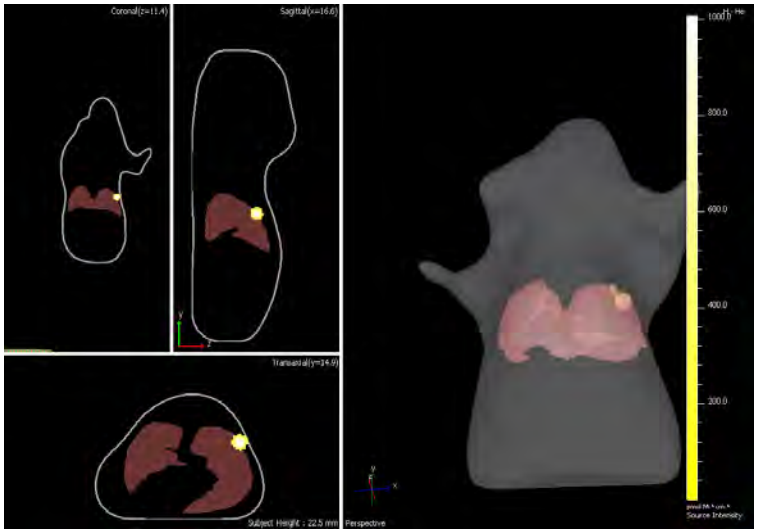
Cetuximab (Erbitux) inhaled in right lung

Nebulisation of Cetuximab tagged with XF680



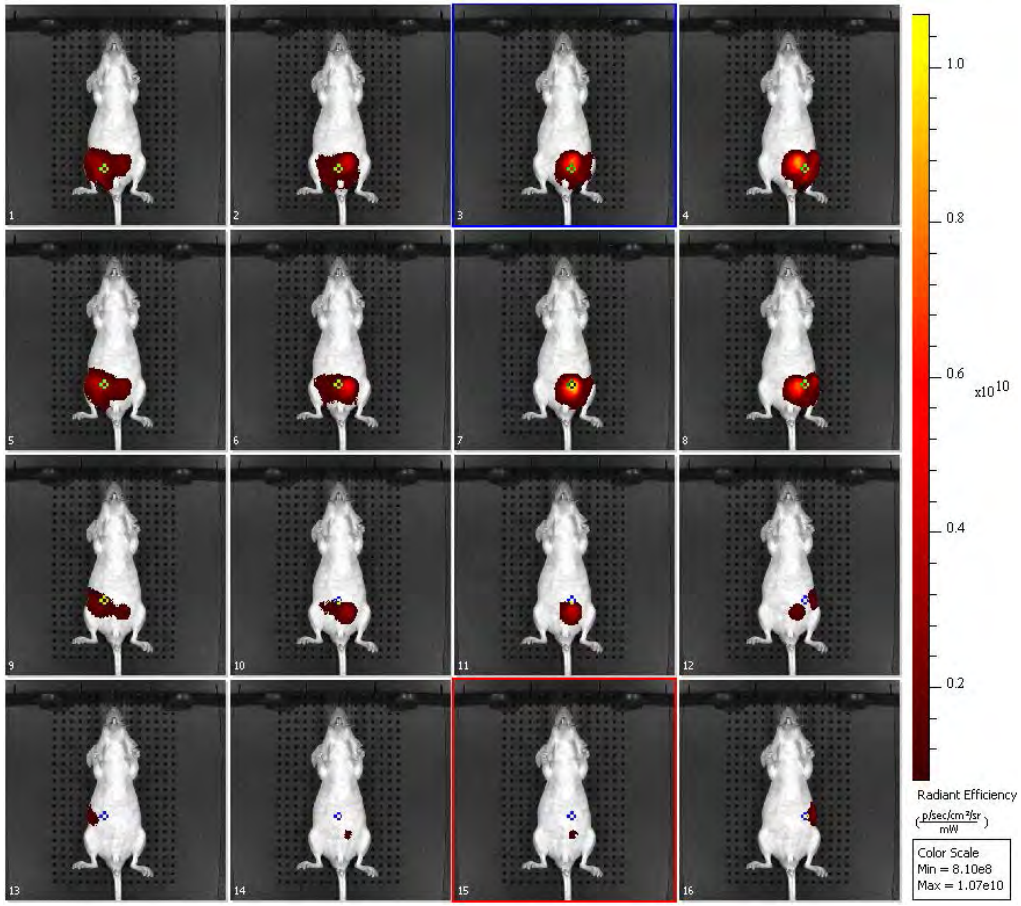






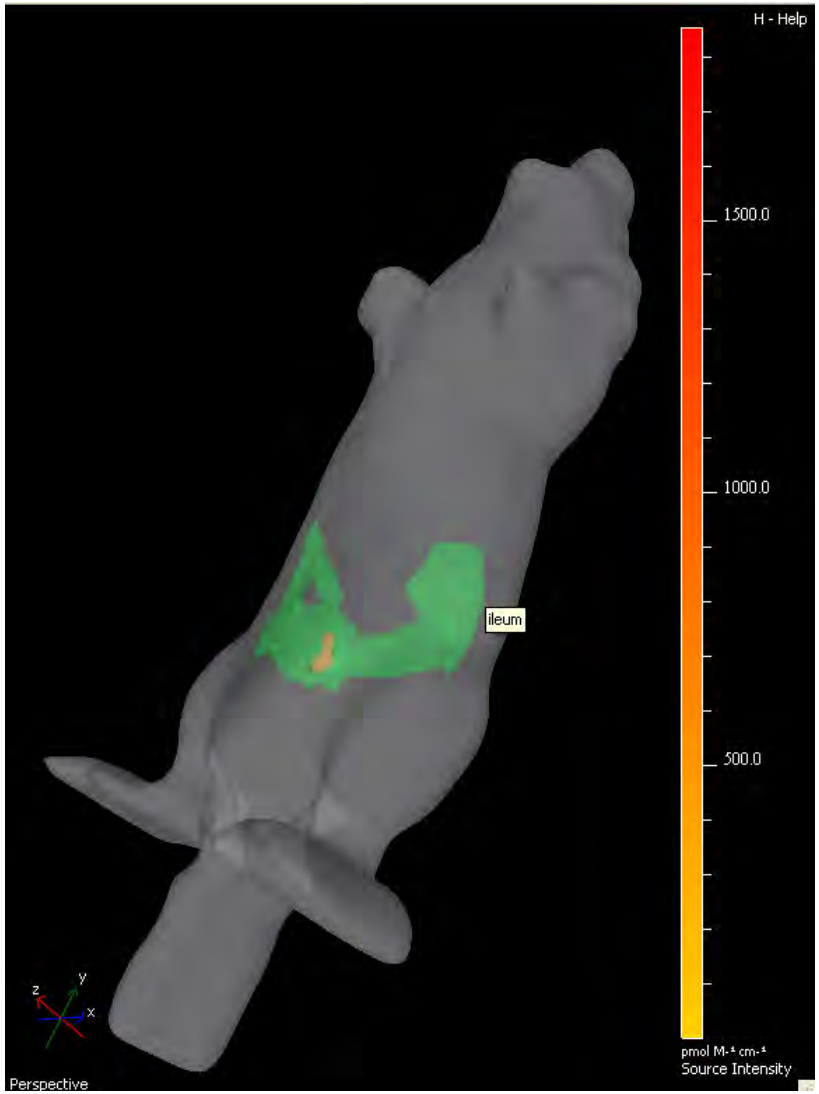
XenoLight 750 Herceptin Conjugate

Ex: 745nm Em: 800 nm



50 μg XLCF750 dye Herceptin conjugate
Injected IV on Day 20
Imaged on Day 22, T=48 hour

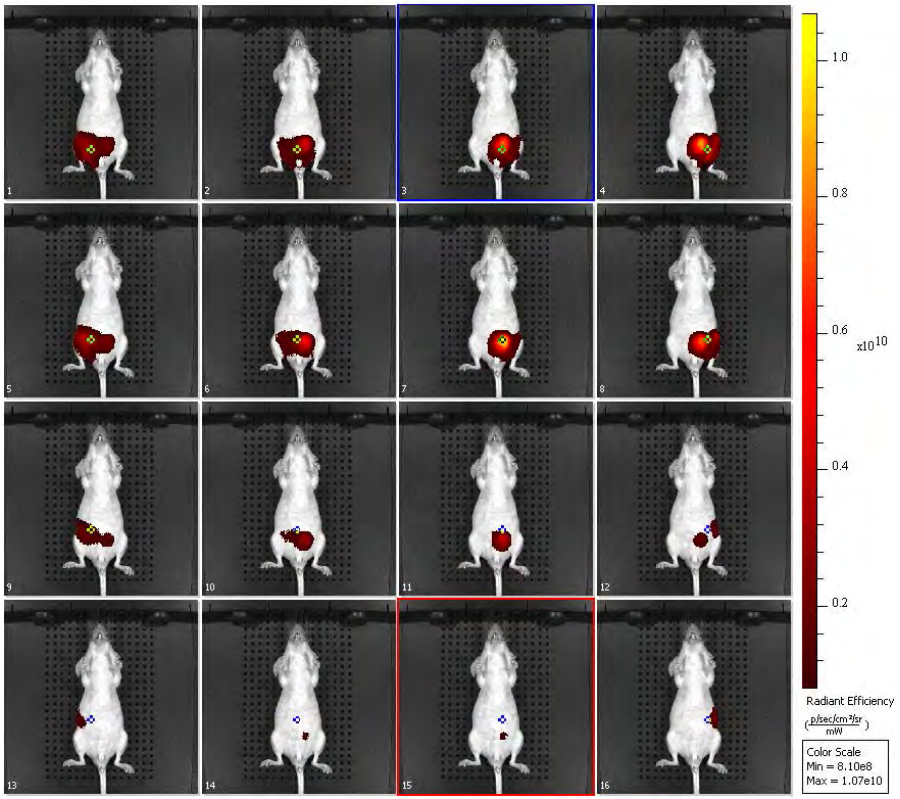
.



In vivo dual modality tomography

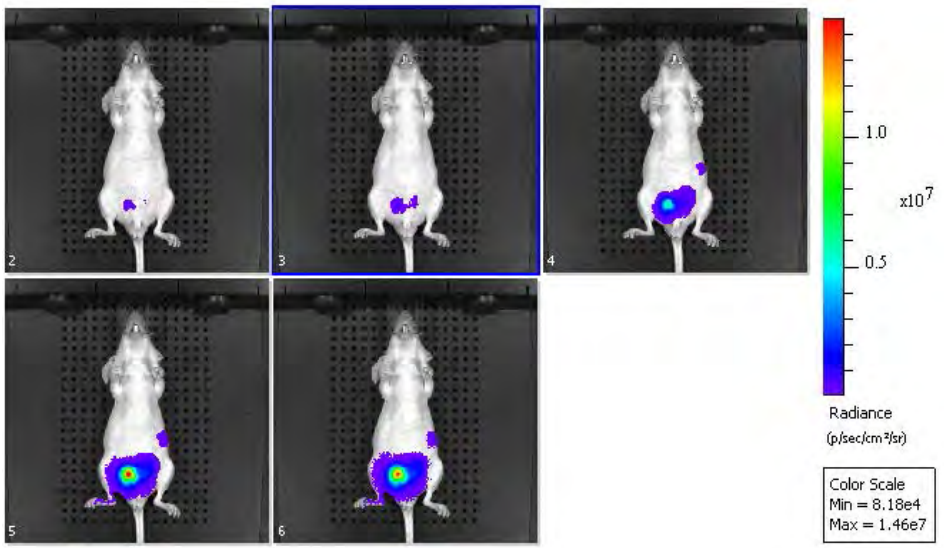
Fluorescence data

Ex: 745nm Em: 800 nm



50 μg XF750 dye Herceptin conjugate
Injected IV on Day 20
Imaged on Day 22, T=48 hour

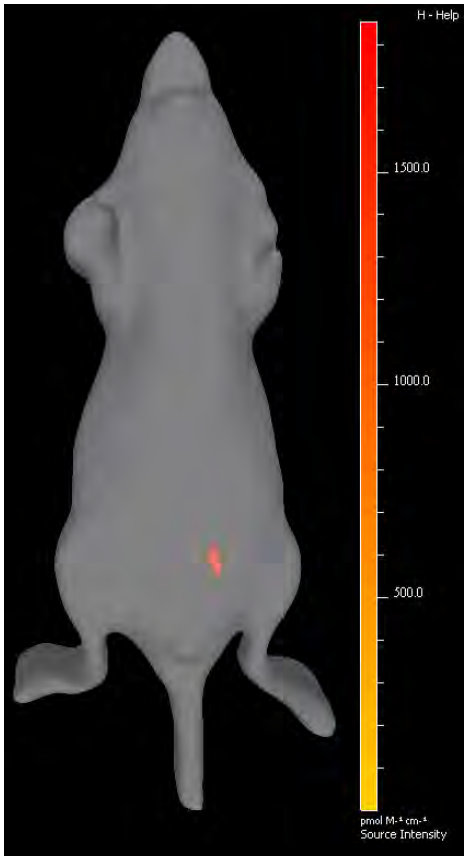
Bioluminescence Data

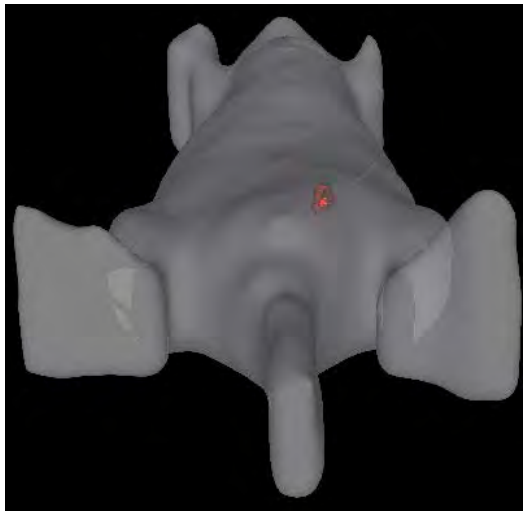


5×10^5 PC3M-luc cells
Injected orthotopically in the prostate
Imaged on Day 22

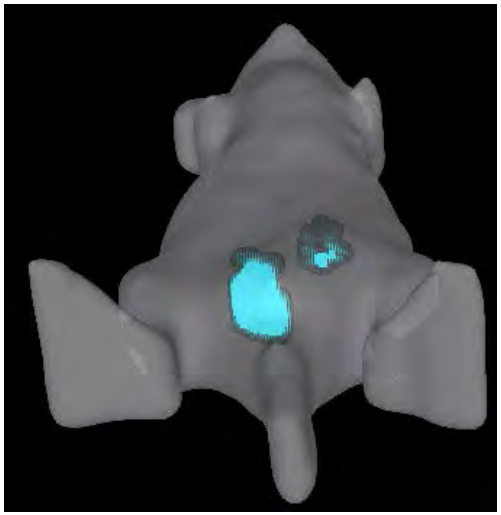
In vivo dual modality tomography

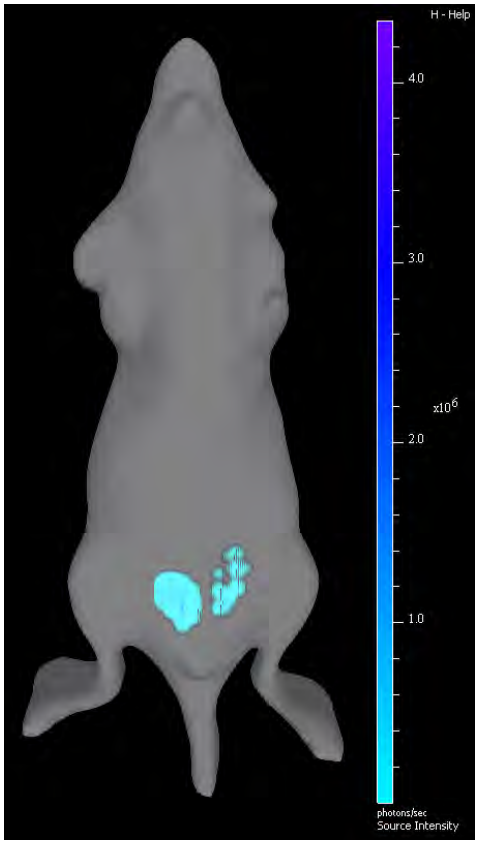
Fluorescence Imaging Tomography - FLIT





Bioluminescence Imaging Tomography - DLIT

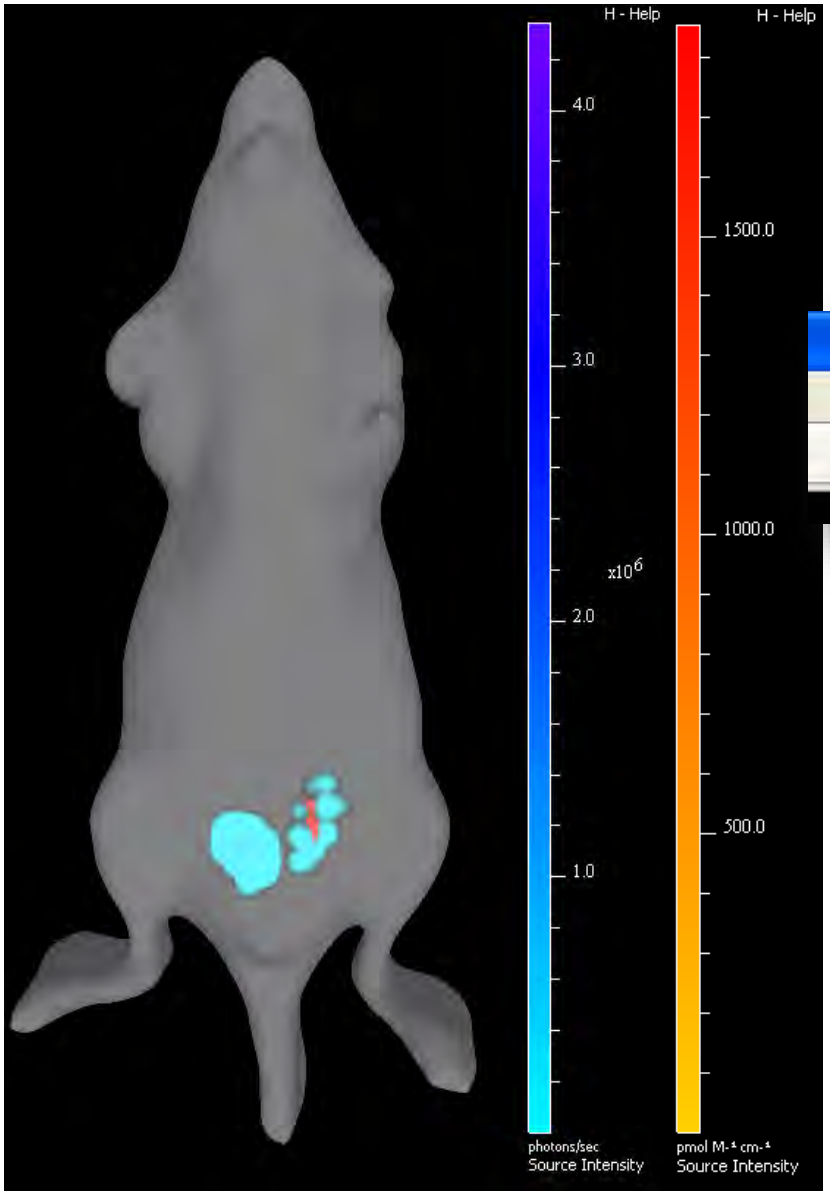


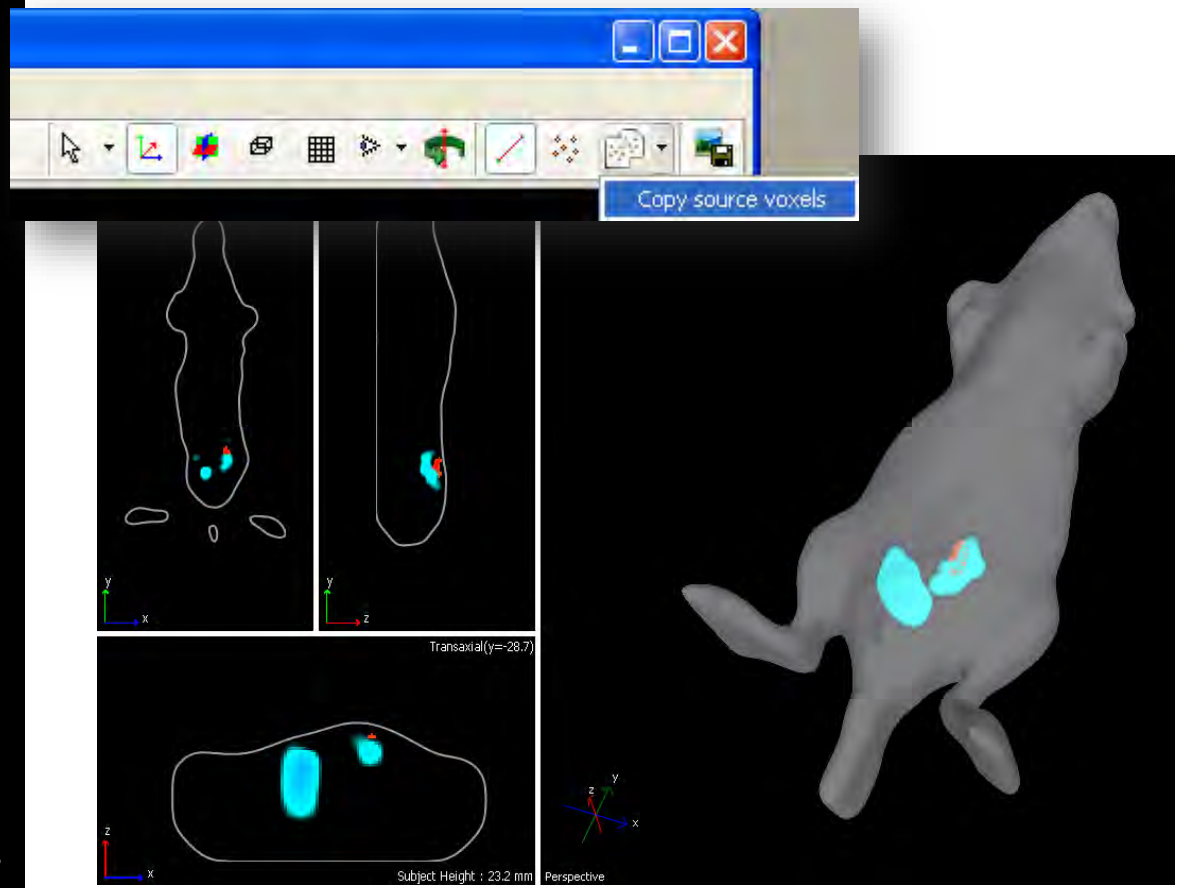


	Mouse Left Tumor Depth [mm]	Mouse Right Tumor Depth [mm]
DLIT	5.8	2.9
FLIT	--	3.1

In vivo Dual Modality Tomography

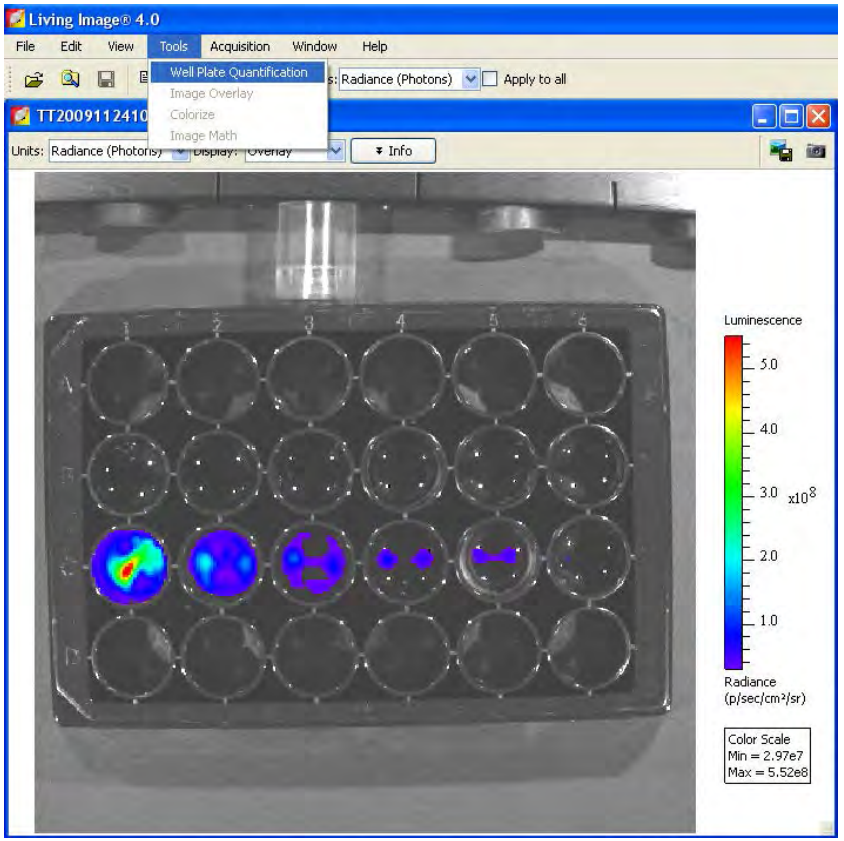
- Copy and paste voxels from FLIT or DLIT reconstructions

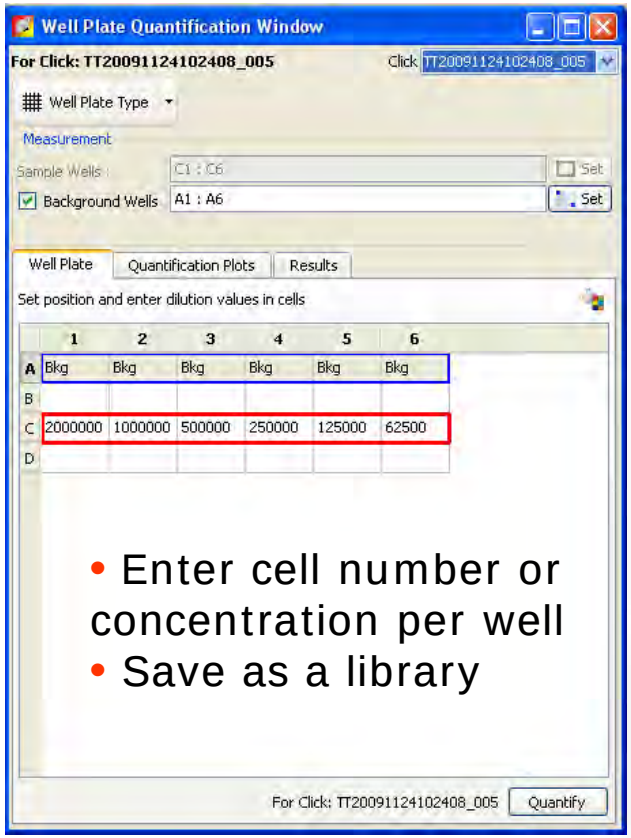




Utilize Well Plate Quantification to Determine Cell Number or pMol of Reporter

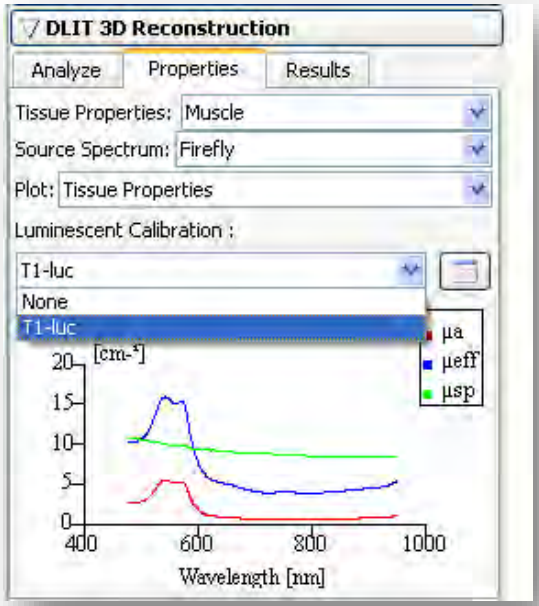
- Dilute your cells or dye and image
- Select Well Plate Quantification from Tools menu

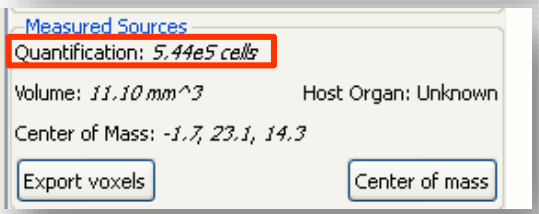




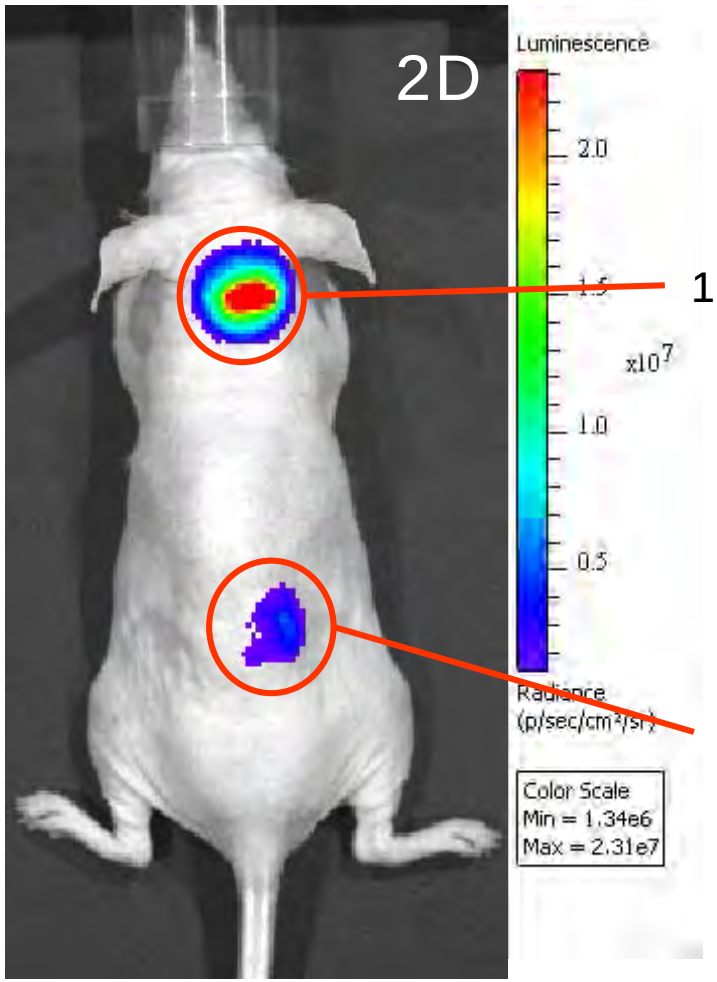
- Enter cell number or concentration per well
- Save as a library

- Choose library when reconstructing





Utilize Well Plate Quantification to Determine Cell Number or pMol of Reporter



5.44e5 cells

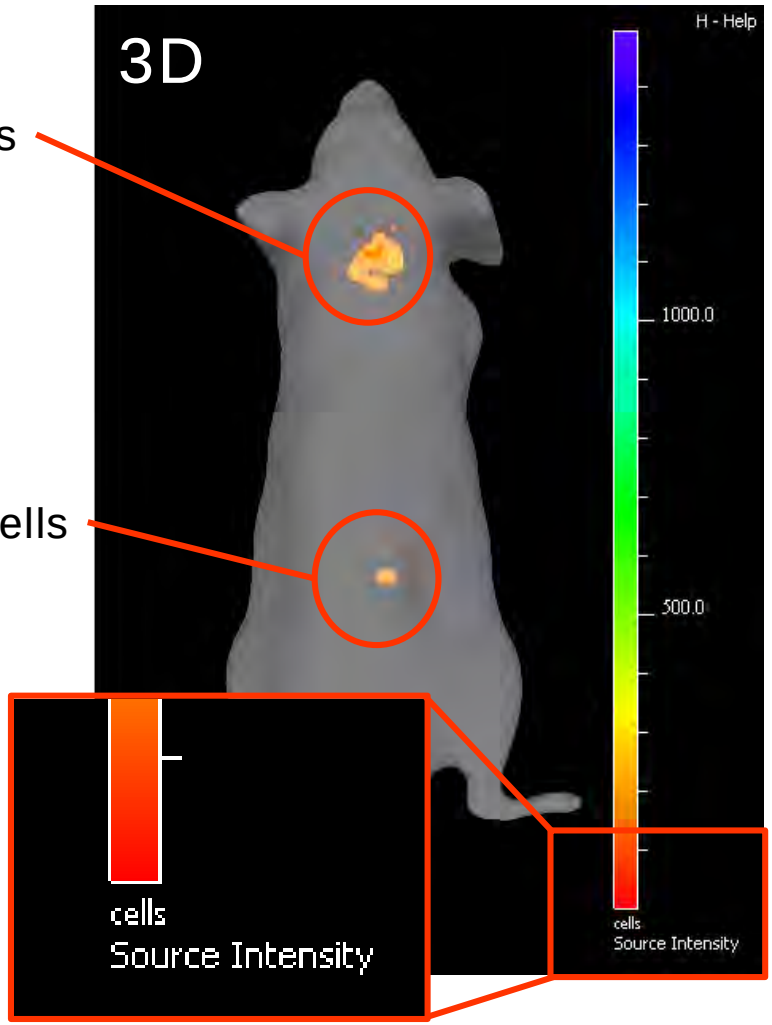
vs.

1.253e8 photons/sec

2.93e4 cells

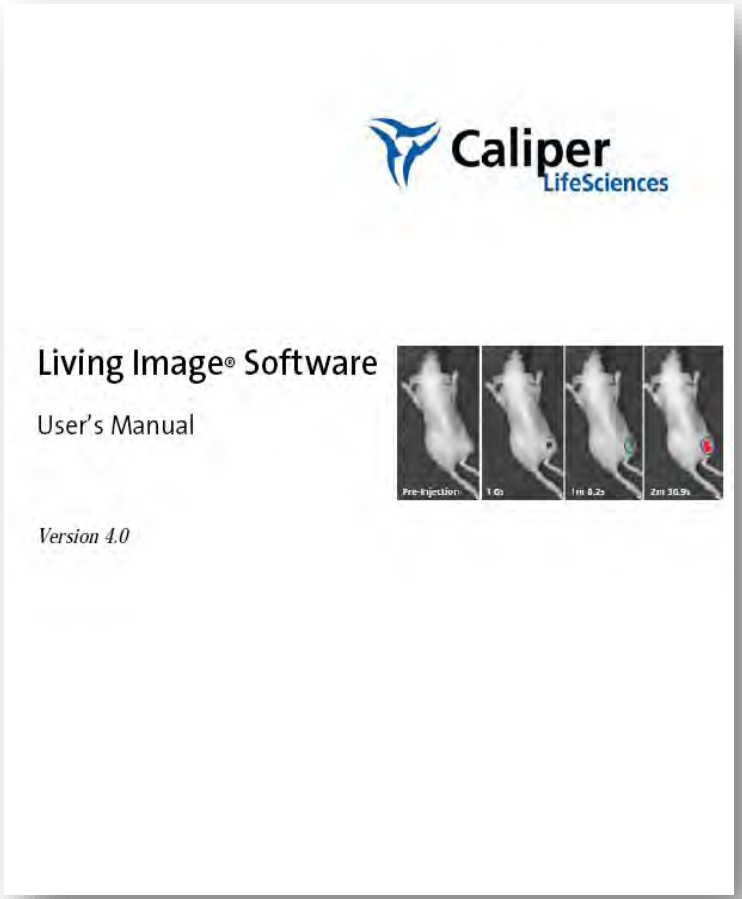
vs.

1.79e7 photons/sec



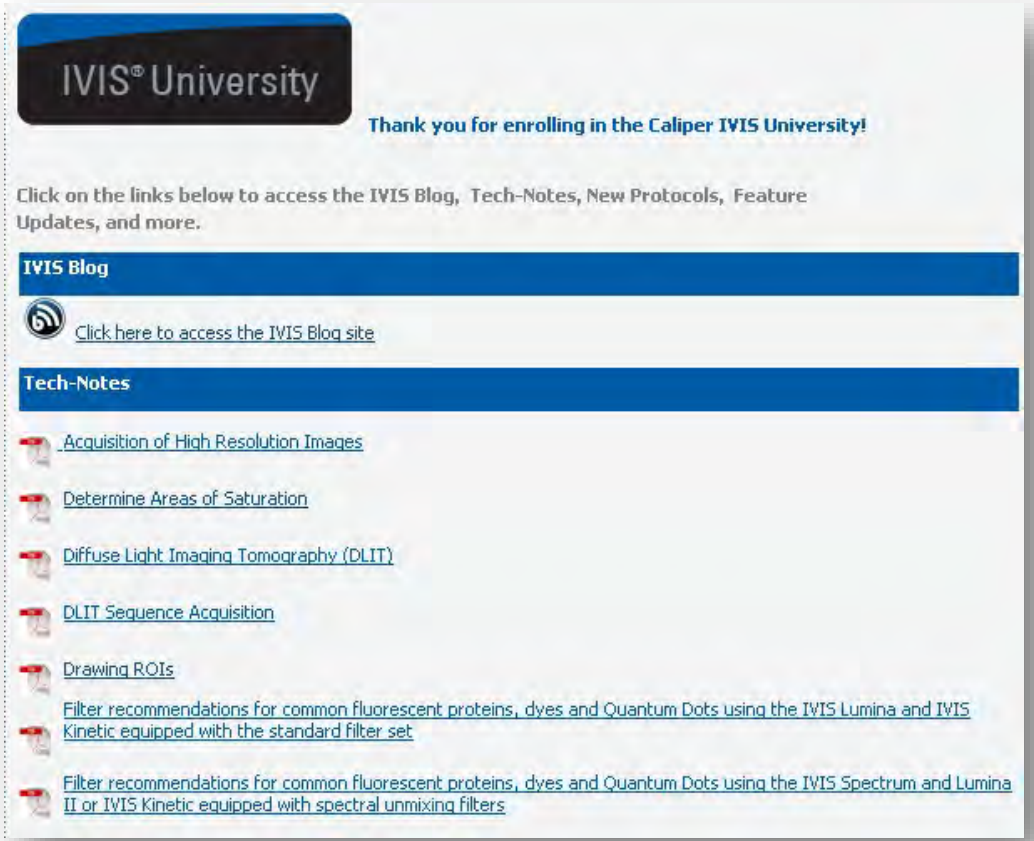
For an In Depth Study

IVIS Software Manual



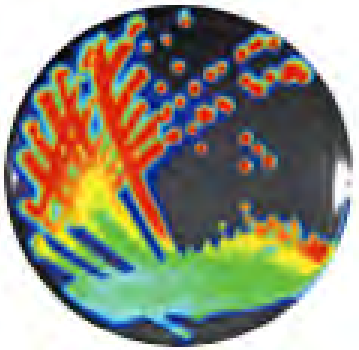
IVIS University Web page

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

Summary

- IVIS Spectrum is a flexible and sensitive instrument for both bioluminescent and fluorescent imaging

- 28 filters cover all bioluminescent and fluorescent probes/reporters of interest for *in vivo* imaging

- Transillumination and spectral unmixing tools improve sensitivity by reducing autofluorescence

- Single view 3D reconstruction tools for bioluminescent and fluorescent imaging

- Tools for co-registration with other imaging modalities available

Thanks for your attention!!

Caliper LifeSciences

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