TECHNICAL NOTE

Determining sensitivity of *in vivo* optical imaging systems.

Highlights

- The IVIS systems can achieve a very low minimum detectable radiance (MDR) of 10 photons/sec/cm^2/sr
- However, MDR measurements can be manipulated and are a poor predictor of success for *in vivo* imaging experiments.
- More meaningful measurements of sensitivity include signal-to-background ratios and minimum number of detectable cells
- Many factors affect the ability to visualize emitted light *in vivo*

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- Ways to measure sensitivity
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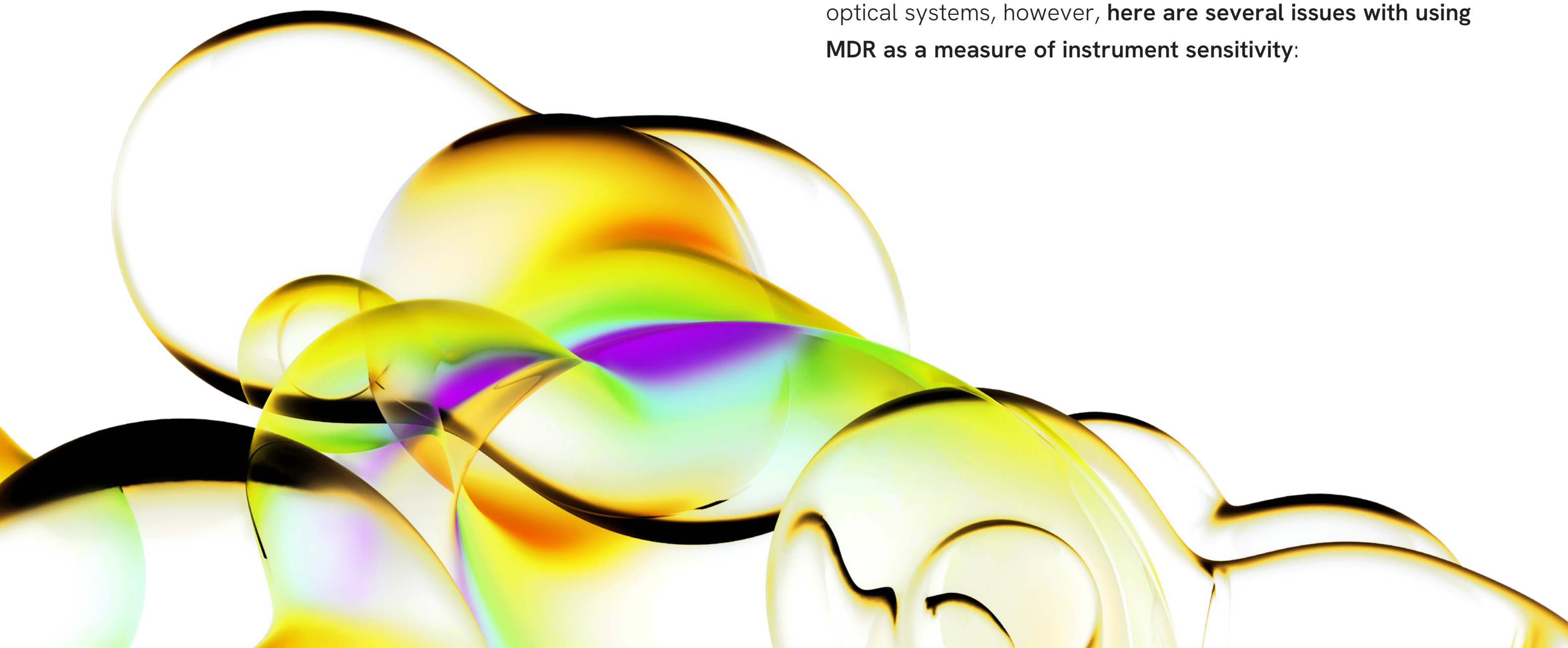
The IVIS™ instruments are Revvity's industry-leading preclinical optical imaging systems with a 20+ year history of sensitive detection of light emission from luminescent or fluorescent sources. The systems can detect light from a wide variety of samples including cells in well plates and ex vivo organs but are designed predominantly with *in vivo* imaging in mind.

Ways to measure sensitivity

There are three commonly used methods to define sensitivity of *in vivo* optical imaging instruments: minimum detectable radiance, signal-to-background ratios, and minimum number of detectable cells. We will review the pros and cons of each method.

Minimum Detectable Radiance (MDR)

MDR is a physical characteristic of the instrument itself, which is independent of properties of the imaged samples. MDR corresponds to the radiance in (photons/sec/cm^2/sr) emitted from the sample surface for which the signal-to-background ratio is equal to 1 for a binned pixel and a given exposure (sr = steradian). It is easy to want a universal measurement to gauge the sensitivity and performance of optical systems, however, **here are several issues with using MDR as a measure of instrument sensitivity**:



1. **MDR values can be manipulated**
   Changing the acquisition settings changes the MDR. For example, if you use an exposure time of 1 hour (never used in practice), you will have a better MDR than if you use an exposure time of 10 seconds (commonly used in practice). Likewise, increasing the image bin size improves the MDR at the expense of decreasing the spatial resolution. For the IVIS Spectrum 2, the lowest measured MDR of 10 photons/sec/cm²/sr used a binning of 64 and a 5 min exposure (see Table 1). However, binning 64 severely pixelates the image data as illustrated in Figure 1, and therefore, is never used. MDR values are highly dependent upon the settings and unrealistic imaging settings can be used to force MDR calculations to lower numbers. Therefore, we caution against relying on MDR as a ground truth measure of sensitivity and predictor of future success.

Table 1: MDR measurements for the IVIS Spectrum 2. Includes measured MDR for a 300 sec exposure time as a function of binning.

Exposure time (sec)	300	300	300	300
Bin size	8	16	32	64
MDR (photon/sec/cm²/sr)	92	42*	23	10

*This MDR value is measured using settings that are used in real *in vivo* imaging experiments.

2. **MDR depends on the instrument's calibration**
   MDR is defined in physical units of radiance and thus requires absolute calibration so an exact relationship between the instrument's camera counts and the radiance emitted by the sample can be determined. The IVIS instruments are thoroughly characterized using custom radiometric sources that are accurately calibrated by NIST (using NIST Photometric Test 34100S).[1]

3. **MDR does not consider background**
   MDR does not consider autoluminescent or autofluorescent background signal. Even with an ultra-low instrument MDR value, if the tissue background is high, you need greater light emission from the reporter to detect it *in vivo*.

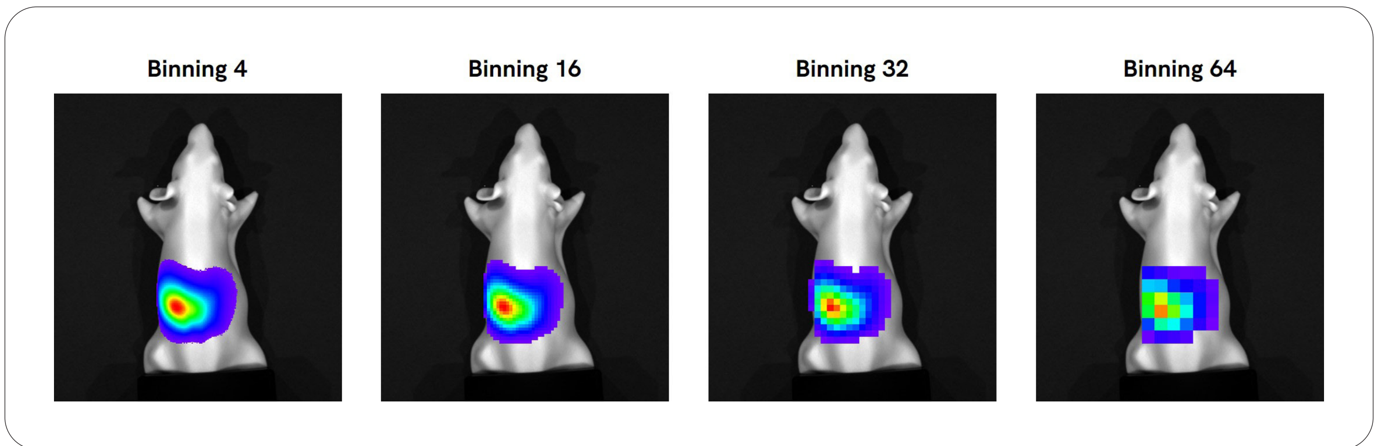


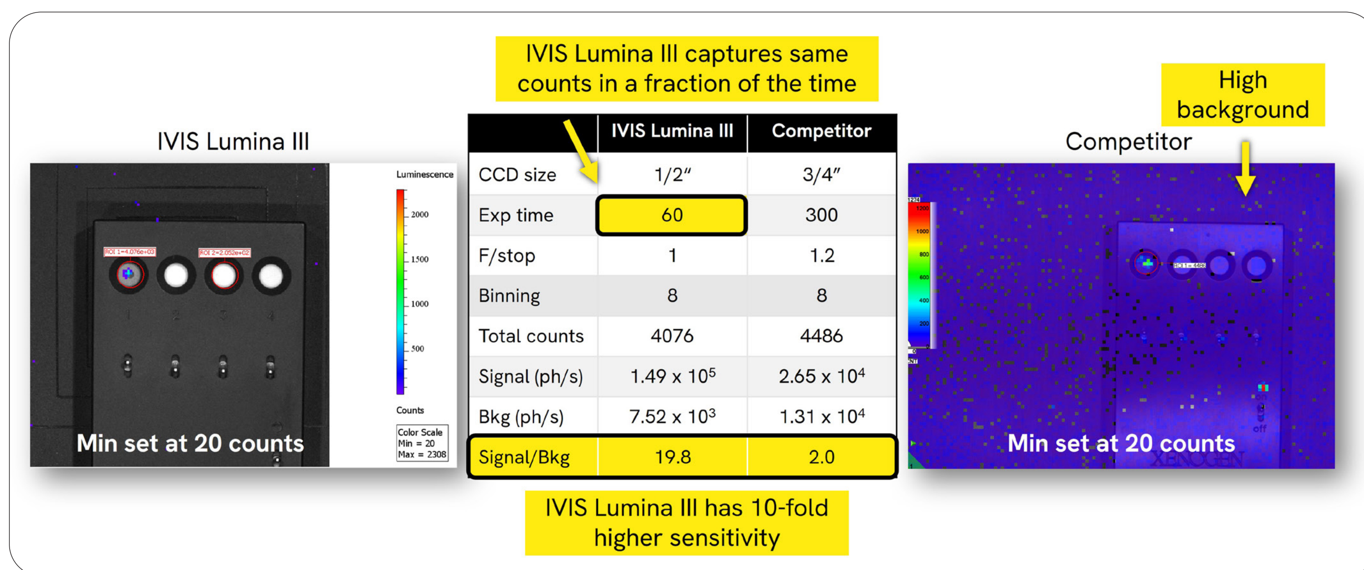
Figure 1. Effects of binning on spatial resolution.

[1]*Calibration of Radiance, NIST Special Photometric Test 37100S". Report of Calibration available upon request.*

Signal-to-Background Ratios (SBR)

SBR is a much better method for determining sensitivity because it considers sources of background and noise which, if high enough, will impede signal detection. Even if the instrument has a very low MDR value, the signal will not be detectable if the background is also high.

In Figure 2, comparable settings were used to capture a bioluminescence image on the IVIS Lumina III instrument and a competitor's instrument. The exposure time was adjusted to capture approximately the same number of counts on both systems. The IVIS was able to capture the same number of counts in less time than the competitor and the background was much lower leading to an overall 10-fold greater sensitivity of the IVIS.



	IVIS Lumina III	Competitor
CCD size	1/2″	3/4″
Exp time	60	300
F/stop	1	1.2
Binning	8	8
Total counts	4076	4486
Signal (ph/s)	1.49×10^5	2.65×10^4
Bkg (ph/s)	7.52×10^3	1.31×10^4
Signal/Bkg	19.8	2.0

Figure 2: Comparing optical instrument sensitivity using signal-to-background ratios (SBR).

Minimum number of detectable cells

This is an excellent metric for biologists looking to understand how many cells they would be able to see *in vivo*. IVIS™ instruments are the most widely published preclinical optical imaging platform on the market (> 27,000 publications as of 2024) and there are multiple peer-reviewed publications showing very low signals detected *in vivo* including the autoluminescence of the mouse itself (see Table 2). Detecting the autoluminescence of mice means that every signal that is higher than the background can be detected! If a signal is lower than the background, no system will be able to detect it. The signal needs to be enhanced by biological means.

Table 2: Peer-reviewed publications showing high sensitivity of Revvity's IVIS imaging systems.

Citation	Instrument	Reporter	Reporter location	Minimum number of cells detected
Kim et al, 2010, PloS One	IVIS Spectrum	FLuc (luc2)	subQ	1
Iwano et al, 2018, Science	IVIS Lumina III	AkaLuc	lung	1

Factors affecting signal visualization

The ability to see visible wavelength light through tissue depends on many factors including those related to the optical reporter, the hardware, and the software.

Reporter properties

1. **Reporter brightness**
   Brightness for bioluminescent reporters is often measured as: photons/second/cell. This unit combines the efficiency of the enzymatic reaction to produce light and also the transduction efficiency. This measurement will increase when you have more efficient luciferases and more copies of luciferase being expressed per cell. Brightness for molecular fluorescent reporters is measured as: extinction coefficient (ε) x quantum yield (Φ). The extinction coefficient describes how efficiently light is absorbed. The quantum yield describes how efficiently the molecule converts absorbed photons into emitted photons. Brightness from fluorescent proteins will also depend on transduction efficiency.

2. **Reporter wavelength**
   Longer wavelength visible light (> 650 nm) will penetrate through tissue better giving optimal results for *in vivo* imaging experiments. Shorter wavelength visible light (< 650 nm) will be attenuated more due to scattering and absorption by endogenous molecules, such as oxyhemoglobin (Figure 3)

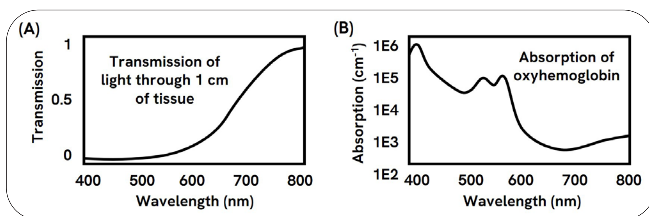


Figure 3: The effect of wavelength on light transmission through tissue (A) and absorption by oxyhemoglobin (B).

3. **Reporter depth**
   Visible light has poor penetration through tissue compared to higher energy photons used by other imaging modalities (e.g., x-ray, CT, and PET). Longer wavelength reporters (> 650 nm) are best for *in vivo* imaging due to lower attenuation by tissue (Table 3).

Table 3: Approximate penetration depths for optical light through tissue.

Light type	Wavelengths (nm)	Approx. Depth (mm)
UV	150-400	< 1
Blue-Green	450-550	1-2
Red	650-800	2-5
Near-IR	800-1200	5-10

4. **Reporter concentration**
   Typically, a higher concentration of reporter = higher emission intensity. Some reporters, for example, indocyanine green (ICG), will self-quench if the concentration gets too high. The max concentration that can be injected will depend upon any self-quenching properties of the reporter and any dose-related toxicity to the animal.

Hardware

1. **Excitation source power**
   This parameter matters only for fluorescence imaging as bioluminescence imaging does not require an excitation light source. Higher lumens = a greater number of incident excitation photons and greater number of emitted fluorescence photons.

2. **Filters**
   This parameter matters only for fluorescence imaging where bandpass filters are used and not for bioluminescence imaging where the emission filter position is open to collect all light. More filter options mean there is a greater likelihood that the bandpass filter applied will capture the peak emission of the optical reporter. Also, broader bandpass filters will capture more light. In addition, some light will be absorbed or scattered by the filter, therefore, the optical transparency of the filters also matters.

3. **Imaging system sensitivity**
   The detector's sensitivity refers to its ability to record very small numbers of photons. Features that affect sensitivity include camera quantum efficiency and optical system transmission across the wavelengths to be used, aperture size (smaller f/numbers = more light captured), physical CCD pixel area (larger pixel area = more light captured per pixel), logical pixel size (i.e., via hardware binning), CCD imager overall system gain (DN/e-) and read noise, and cooling (cooler camera = lower dark current background noise). The IVIS detectors use a 1" back-illuminated CCD chip and are cooled to -90°C providing ultra-sensitive acquisition of luminescent and fluorescent light emission.

Sources of background signal

If an image has high background, the signal of interest will be difficult to see. In bioluminescence imaging, background signal is primarily caused by tissue autoluminescence and the diffuse scattered light from the luminescent source itself. In fluorescence imaging, additional background sources include emission from the instrument itself, from tissue autofluorescence, from plant material (e.g., chlorophyll) or other fluorescent material in the animal's diet, and from the excitation source.

Spectral unmixing, a software tool within Living Image™, is the most efficient way to remove the autofluorescent background which will also enhance sensitivity. Revvity's spectral unmixing technology is the most advanced method on the market (see our White Paper: The "why" and "how" of spectral unmixing.) For example, Figure 4 shows a mouse with weak dihydroethidium (DHE) signal in the brain. It was imaged with a series of 20 nm bandpass filters and the autofluorescence background signal is about twice as high as the DHE signal. However, after spectral unmixing, the SBR increased from 0.5 to 22 which made the DHE signal clearly visible in the unmixed image.

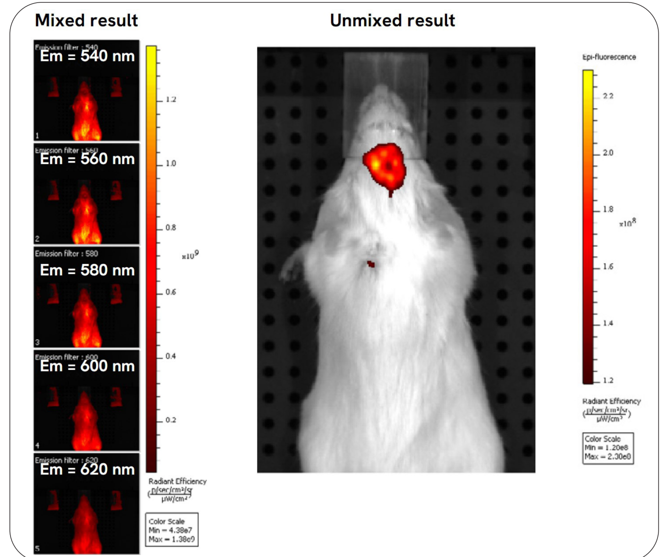
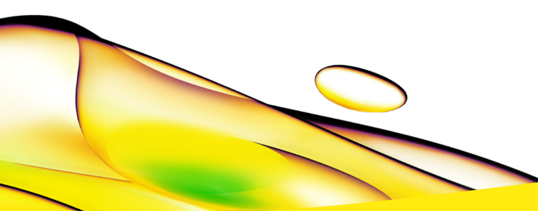


Figure 4: Spectral unmixing improves visualization and sensitivity of fluorescence images.
Left: before unmixing, Right: after unmixing.

Conclusion

- MDR is not the best measure of an instrument's sensitivity because it can be manipulated and does not consider background signal.
- Better methods for evaluating sensitivity are signal-to-background ratios and minimum number of detectable cells.
- Multiple factors affect the ability to see signal in an animal, including properties of the optical reporter, hardware components, and software features like spectral unmixing. All must be considered when designing a new *in vivo* imaging experiment.



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