



Luminescent Background Sources and Corrections

The background sources of light from luminescent images are inherently very low. This appendix discusses sources of background and how to manage them. Due to the extreme sensitivity of the IVIS Imaging System, residual electronic background (dark current) and luminescent emission from live animals (autoluminescence) are measurable and must be taken into account.

For information on fluorescent background, see *Concept Tech Note 4 – Fluorescent Imaging*.

Electronic Background

The cooled CCD camera used in an IVIS Imaging System has electronic background that must be accurately measured and subtracted from the image data before the light intensity is quantified. Raw data that is not corrected for electronic background results in erroneous ROI measurements. Incorrect background subtraction may also result in serious errors. However, it is not necessary to subtract the electronic background when making a simple visual inspection of an image.

The types of electronic background include:

- *Read bias* - An electronic offset that exists on every pixel. This means that the zero photon level in the readout is not actually zero, but is typically a few hundred counts per pixel. The read bias offset is reproducible within errors defined by the *read noise*, another quantity that must be determined for quantitative image analysis.
- *Dark current* - Electronic background generated by the thermal production of charge in the CCD. To minimize dark current, the CCD is cooled during use.

Read Bias & Drift

Prior to a luminescent image exposure, the Living Image software initiates a series of zero-time exposures (*image readout*) to determine a read bias measurement.

If a dark charge background is available for the luminescent image, the average bias offset for the read bias image stored with the dark charge measurement is compared to the average bias offset determined with the read bias measurement made prior to the image. The difference, or *drift correction*, is stored with the luminescent image data, and is later used to correct minor drift (typically less than two counts/pixel) that may occur in the bias offset since measuring the dark charge background.

If a dark charge background is not available at the time of the luminescent image exposure, the software checks to see if the selected image parameters warrant a dark charge measurement (large binning and long exposure time). If a dark charge image is not required, the read bias will be used. If a dark charge is recommended, the software provides the option of using the read bias measurement instead. Since the read bias is by far the largest component of background, using a read bias measurement instead of a dark charge measurement is often acceptable. If read bias is used instead of a dark charge background, the read bias image is stored with the image data rather than the usual background information.

If the amount of dark charge associated with an image is negligible, read bias subtraction is an adequate substitute for dark charge background subtraction. Dark charge increases with exposure time and is more significant at higher levels of binning. A good rule of thumb is that dark charge is negligible if:

$$\tau B^2 < 1000$$

where τ is the exposure time (seconds) and B is the binning factor.

Under these conditions, dark charge contributes less than 0.1 counts/pixel and may be ignored.

Dark Charge

Dark charge refers to all types of electronic background, including dark current and read bias. Dark charge is a function of the exposure time, binning level, and camera temperature. A dark charge measurement should be taken within 48 hours of image acquisition and the system should remain stable between dark charge measurement and image acquisition. If the power to the system or camera controller (a component of some IVIS Imaging Systems) has been cycled or if the camera temperature has changed, a new dark charge measurement should be taken.

The dark charge is measured with the camera shutter closed and is usually performed automatically overnight by the Living Image software. The software acquires a series of zero-time exposures to determine the bias offset and read noise, followed by three dark exposures. The dark charge measurement usually takes more than three times as long to complete as the equivalent luminescent exposure.

Background Light On the Sample

An underlying assumption for *in vivo* imaging is that all of the light detected during a luminescent image exposure is emitted by the sample. This is not accurate if there is an external light source illuminating the sample. Any reflected light will be detected and is indistinguishable from emission from the sample.

The best way to deal with external light is to physically eliminate it. There are two potential sources of external light: a light leak through a crack or other mechanical imperfection in the imaging chamber or a source of external illumination.

IVIS Imaging Systems are designed to be extremely light tight and are thoroughly checked for light leaks before and after installation. Light leaks are unlikely unless mechanical damage has occurred. To ensure that there are no light leaks in the imaging chamber, conduct an imaging test using the High Reflectance Hemisphere (Figure 1).

A more subtle source of external illumination is the possible presence of light emitting materials inside the imaging chamber. In addition to obvious sources such as the light emitting diodes (LEDs) of electronic equipment, some materials contain phosphorescent compounds.



NOTE: Do not place equipment that contains LEDs in the imaging chamber.

Phosphorescence is a physical process similar to fluorescence, but the light emission persists for a longer period. Phosphorescent materials absorb light from an external source (for example, room lights) and then re-emit it. Some phosphorescent materials may re-emit light for many hours. If this type of material is introduced into the imaging chamber, it produces background light even after the chamber door is closed. If the light emitted from the phosphorescent material illuminates the sample from outside of the field of view during imaging, it may be extremely difficult to distinguish from the light emitted by the sample.

IVIS® Imaging Systems are designed to eliminate background interference from these types of materials. Each system is put through a rigorous quality control process to ensure that background levels are acceptably low. However, if you introduce such materials inadvertently, problems may arise.

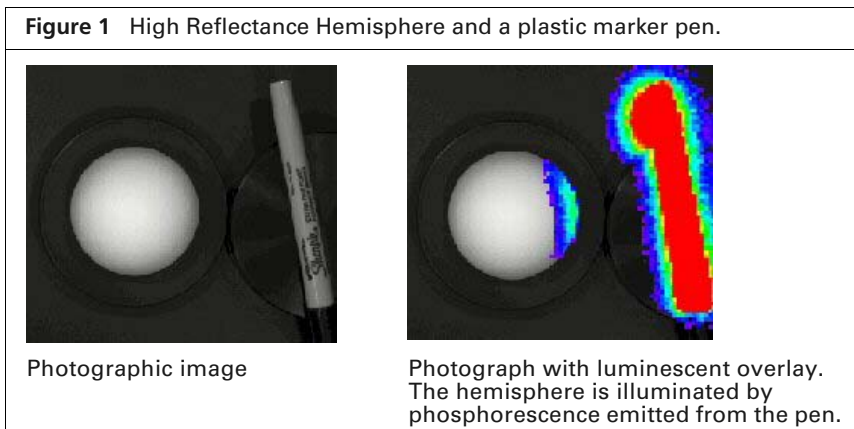
Problematic materials include plastics, paints, organic compounds, plastic tape, and plastic containers. Contaminants such as animal urine can be phosphorescent. To help maintain a clean imaging chamber, place animal subjects on black paper (for example, Artagain black paper, Strathmore cat. no. 445-109) and change the paper frequently. Cleaning the imaging chamber frequently is also helpful.

! **IMPORTANT:** Use only cleaning agents approved by Caliper. Many cleaning compounds phosphoresce! Contact Caliper technical support for a list of tested and approved cleaning compounds.

If it is necessary to introduce suspect materials into the imaging chamber, screen the materials by imaging them. Acquire an image of the material alone using the same settings (for example, FOV and exposure time) that will be used to image the sample to determine if the material is visible in the luminescent image.

Microplates (white, black, or clear plastic) can be screened this way. Screen all three types with a test image. White plates appear extremely bright by IVIS Imaging System standards and interfere with measurements. Black or clear plastic microplates do not phosphoresce, making them better choices.

The High Reflectance Hemisphere provides a more definitive way to determine the presence of an undesirable light source (Figure 1). It is a small white hemisphere that is coated with a non-phosphorescent material. A long exposure image of the hemisphere should produce a luminescent image in which the hemisphere is not visible.



If any part of the hemisphere exhibits what appears to be luminescent emission, it is actually the light reflected from a source illuminating the hemisphere. Observe the side of the hemisphere that is illuminated to help determine the source location.

In Figure 1, the pen appears very bright due to phosphorescent emission that is also illuminating the portion of the hemisphere next to the pen. If the pen had been outside the field of view, it would not have been imaged, and the source of the phosphorescence would be less obvious. However, the illumination of the hemisphere would still be very apparent and indicative of a light pollution problem.

! **IMPORTANT:** Handle the High Reflectance Hemisphere by its black base plate while wearing cotton gloves provided by Caliper. Skin oils can phosphoresce and will contaminate the hemisphere. Latex gloves and the powder on them may also phosphoresce. If the hemisphere becomes contaminated, contact Caliper technical support for a replacement. There are no known agents that can clean the hemisphere. To check the hemisphere for contamination, take several images of the hemisphere, rotating it slightly between images. A glowing fingerprint, for example, will rotate with the hemisphere, while a glowing spot due to external illumination most likely will not.

Background Light From the Sample

Another source of background is the natural light emitted from a sample that is not due to emission from the source of interest in the sample. This type of background may be due to a material associated with the experimental setup. For example, the cell culture medium may phosphoresce. Materials should be screened so you can identify and eliminate problematic materials. If a background source is phosphorescent and the phosphorescent lifetime is relatively short, you can try keeping the sample in the dark for a long period before imaging to reduce background light emission.

Occasionally there is no way to eliminate the natural light emission of the sample. The natural light emission associated with living animals (*autoluminescence*) is a major area of interest in *in vivo* luminescent imaging. Most animals exhibit a low level of autoluminescence. Usually this is only a problem when looking for very low signals at the highest levels of sensitivity.

Caliper has conducted tests to try to minimize the source of the background light emission in mice.

Test Description	Observation
Test 1: Subject animals were housed in the dark 12 hours prior to imaging.	Background emission levels were not reduced. A phosphorescent component in mouse fur or skin is not the source of light emission.
Test 2: White-furred animals were shaved prior to imaging	No increase or decrease in background emission levels.
Test 3: Alfalfa (known to be phosphorescent) was eliminated from the animal diet.	An alfalfa-free diet reduced background emission slightly, but not significantly.

The sources of autoluminescence are not yet fully understood. No external sources have been proven to cause natural light emissions, so it is possible that a chemiluminescent process associated with metabolic activity in living animals is the source of animal background. This is supported by the observation that the level of background light drops significantly in euthanized animals.

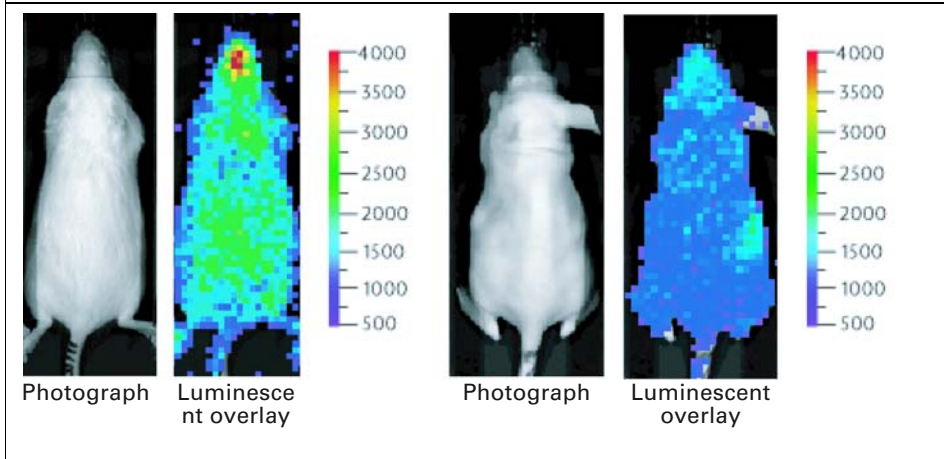
In [Figure 2](#), the background light emission is clearly visible in the images of a control white-furred mouse and a nude mouse. The images are five minute, high-sensitivity (high binning) exposures. The average emission from a white-furred mouse and a nude mouse is approximately 1600 photons/s/cm²/sr and 1000 photons/s/cm²/sr, respectively. Since these values are well above the lower limit of detection of the IVIS Imaging System (~100 photons/s/cm²/sr), the background light emission from the mouse determines the limit of detection.

An approximation of this background (determined by making similar measurements on either control animals or regions of the subject animal that do not contain the primary signal) can be subtracted from ROI measurements.

Note that the background light emission is not uniform over the entire animal. In [Figure 2](#), images of control animals (mice) show a somewhat higher background component originating from the abdominal and thoracic regions. Therefore, care must be taken when selecting a representative background area.

Figure 2 Background light emission

Background light emission from a female white furred (Swiss Webster) (left) and a female nude (Nu/nu) mouse (right).



Usually only very low signals at the highest level of sensitivity require this type of background subtraction. For more information on how best to handle these types of measurements, please contact Caliper technical support.