# Image Display and Measurement

## Image Data

### Scientific Image Data

Scientific image data is a two-dimensional array of numbers. Each element of the array (*pixel*) is associated with a number that is proportional to the light intensity on the element. A charge coupled device (CCD) camera used for scientific imaging is essentially an array of photo-sensitive pixels and each pixel collects photons during an image exposure.

The subsequent electronic readout provides a photon intensity number associated with each pixel. In a bright area of the image, more photons are detected and the photon intensity number is greater than the number in a dim area of the image.

The image data can be visualized in different ways, including pseudocolor images (generated by the Living Image software), contour plots, or isometric displays.

### Graphic Image Data

A graphic image is a two-dimensional array of pixels with a color assigned to each pixel. There are several schemes for digitally storing the images. For example, a common scheme assigns a red-green-blue (RGB) color code to each pixel. The RGB code defines how much of each color to apply in order to create the final pixel color. Color photographs or color screenshots are examples of RGB images.
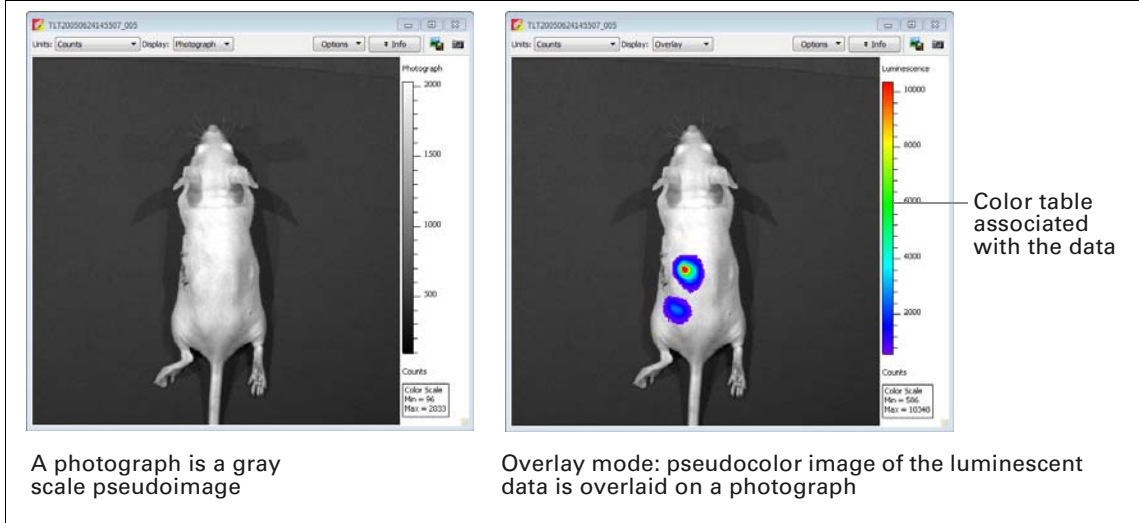
An RBG image is also a two-dimensional array of numbers, but unlike a scientific image, the numbers are only color codes and are not related to light intensity. A graphic image can be exported to a graphic display application.

### Pseudocolor Images

An image can be generated from scientific image data by assigning a color to each numerical value and plotting the array so that each pixel is filled with the color that corresponds to its numerical value. A *color table* defines the relationship between the numerical data and the displayed color. For example, a grayscale color table assigns black to the smallest number in the array, white to the largest number, and shades of gray to the values in between (Figure 1). The resulting image is equivalent to a black and white photograph. An illuminated photographic image acquired on an IVIS Imaging System is an example of a grayscale pseudoimage.

The reverse rainbow color table is also commonly used and assigns violet to the smallest number on the array, red to the largest number, and all of the spectral colors of the rainbow to the values in between (Figure 1).

**Figure 1** Example pseudoimages

A photograph is a gray scale pseudoimage

Overlay mode: pseudocolor image of the luminescent data is overlaid on a photograph

Color table associated with the data

A pseudocolor scheme is typically used to display the numerical contents of scientific image data like the luminescent or fluorescent images acquired on an IVIS Imaging System. The pseudocolor scheme makes it easy to see areas of bright light emission. The amount of light emission can be quantified using measurement ROIs.

Measurement data is independent of the colors displayed in the pseudocolor image. You can change the appearance of the image data without affecting the underlying numeric pixel values. For example, you apply a different color table to the data or adjust the range of numeric values associated with the color table. Measurements that quantify pixel data produce the same results independent of the appearance of the pseudocolor display.

A pseudocolor image can be converted to an RGB color code and saved as an RGB image. The RGB image looks like a pseudocolor image, but does not include the numerical information derived from the light detected in each pixel. Therefore, the amount of light in an RGB image cannot be quantified.

## Overlays

In the overlay display mode, the pseudocolor luminescent or fluorescent image is displayed over the associated grayscale photographic image (Figure 1). Pixels in the luminescent or fluorescent image that are less than the minimum color table setting are not displayed. As a result, the lowest intensity color in the table is transparent and this enables you to view the underlying photographic image in regions where the luminescent light emission is low. While the pixels less than the minimum color table setting are not displayed, they still exist in the image data.

# Quantifying Image Data

The Living Image software can quantify and display scientific image data for several types of measurements.

**Table 1**  Data display units

| Data Display | Description | Recommended For: |
|---|---|---|
| Counts | An uncalibrated measurement of the photons incident on the CCD camera. | Image acquisition to ensure that the camera settings are properly adjusted. Proper image parameter adjustment should avoid image saturation and ensure sufficient signal (greater than a few hundred counts at maximum). |
| Radiance (photons) | A calibrated measurement of the photon emission from the subject. Radiance is in units of "photons/second/cm$^2$/steradian". | Luminescence measurements |
| Radiant Efficiency (fluorescence) | Epi-fluorescence - A fluorescence emission radiance per incident excitation power. <br><br>Transillumination fluorescence - Fluorescence emission radiance per incident excitation power | Fluorescence measurements |
| Efficiency (epi-fluorescence) | Fluorescent emission normalized to the incident excitation intensity (radiance of the subject/illumination intensity) | Epi-fluorescence measurements |
| NTF Efficiency | Fluorescent emission image normalized to the transmission image which is measured with the same emission filter and open excitation filter. | Transillumination fluorescent measurements |

## Counts

When image data is displayed in counts, the image pixel contents are displayed as the numerical output of the charge digitizer on the charge coupled device (CCD) (Figure 2). The counts measurement (also known as *analog digitizer units* (ADU) or *relative luminescence units* (RLU)) is proportional to the number of photons detected in a pixel.

Counts are uncalibrated units that represent the raw amplitude of the signal detected by the CCD camera. A signal measured in counts is related to the photons incident on the CCD camera. The signal varies, depending on the camera settings (for example, integration time, binning, f/stop, or field of view setting).

All IVIS® Imaging Systems include a CCD digitizer that is a 16-bit device, which means that the signal count range is from zero to 65,535. Sometimes the displayed signal count may appear outside of this range due to corrections applied to the image data (for example, background corrections).
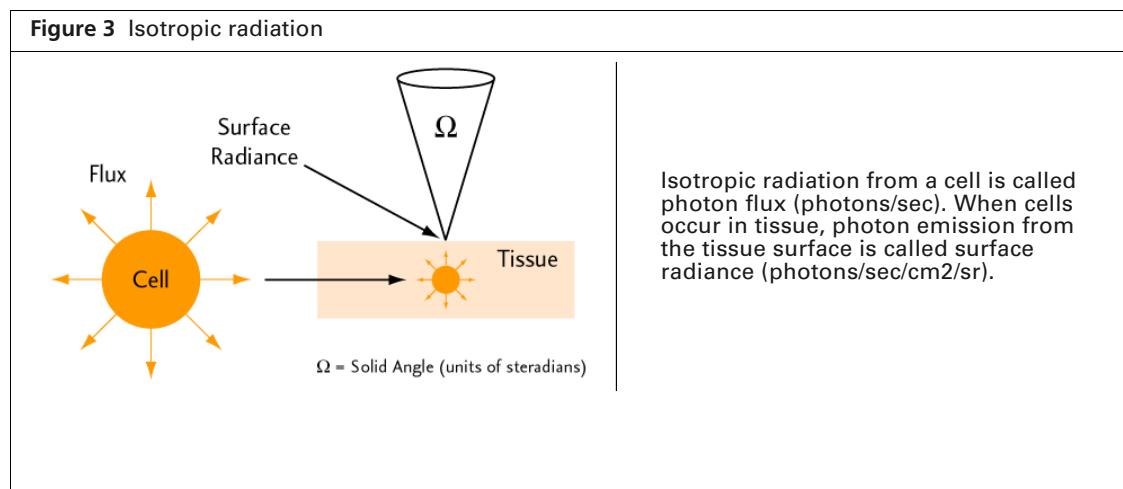
**Figure 2** ROI measurements (counts mode)
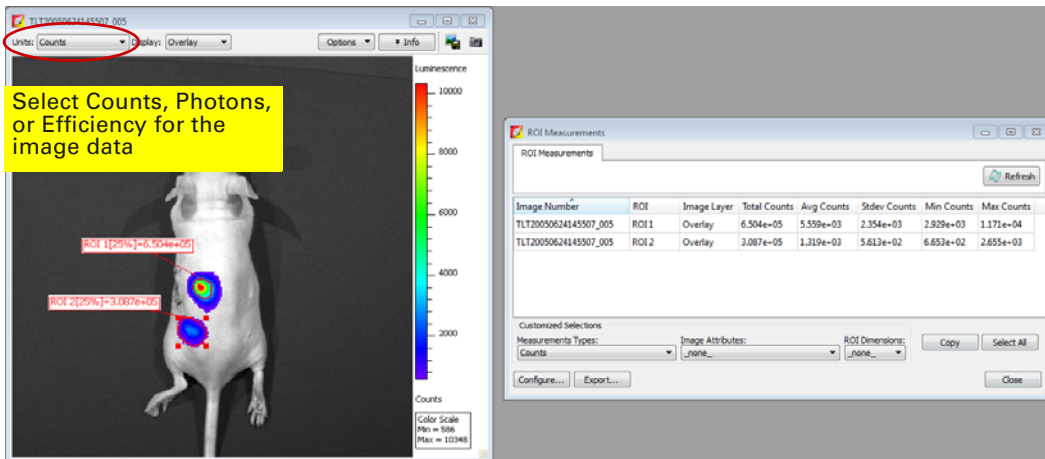


In counts mode, the ROI measurements table includes:
- Total Counts – Sum of all counts for all pixels inside the ROI
- Average Counts – Total Counts/Number of pixels or super pixels
- Quantity ROI Pixels – Number of binned pixels inside the ROI
- Area (CCD pixels) – Number of unbinned CCD pixels inside the ROI

## Radiance

When image data is displayed in radiance units ('photons' for short), the *photon emission from the subject* is displayed in photons/sec/cm²/sr. Counts are a relative measure of the photons incident on the CCD camera and radiance is in absolute physical units that measure the photon emission from the subject.

The radiance unit of photons/sec/cm²/sr is the number of photons per second that leave a square centimeter of tissue and radiate into a solid angle of one steradian (sr) (Figure 3).

**Figure 3** Isotropic radiation



Isotropic radiation from a cell is called photon flux (photons/sec). When cells occur in tissue, photon emission from the tissue surface is called surface radiance (photons/sec/cm2/sr).

A steradian can be thought of as a three-dimensional cone of light emitted from the surface that has a unit solid angle. Much like a radian is a unit of arc length for a circle, a steradian is a unit of solid angle for a sphere. An entire sphere has $4\pi$ steradians. Lens systems typically collect light from only a small fraction of the total $4\pi$ steradians.
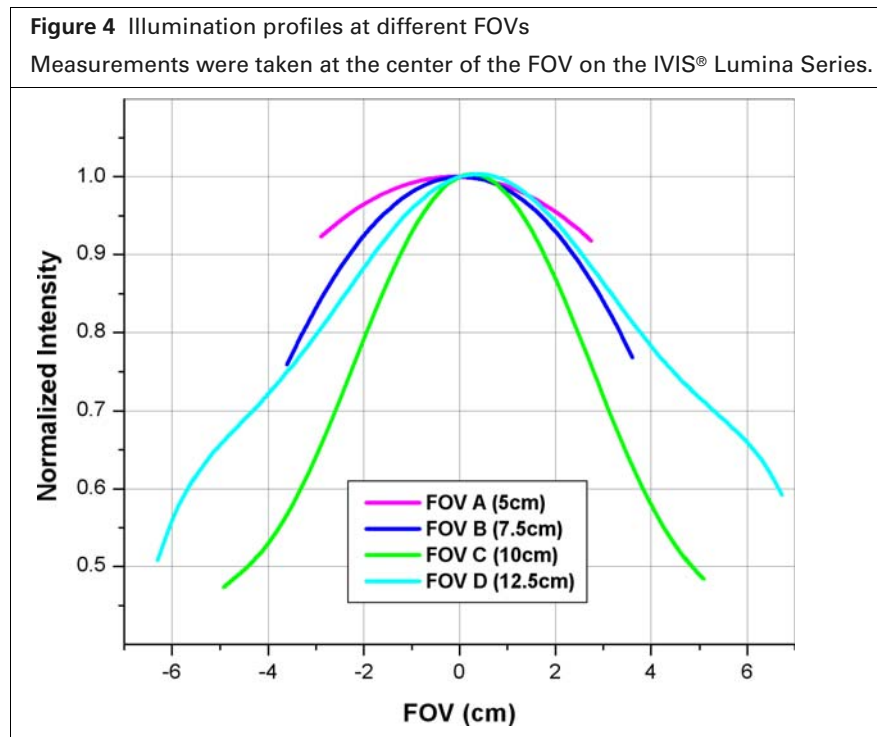
When image data is displayed in radiance mode, the units change to photons/sec/cm$^2$/sr. These are units of photon radiance on the surface of the animal. A very important distinction between these absolute physical units and the relative units of counts is that the radiance units refer to photon emission *from the subject animal itself,* as opposed to counts that refers to a measurement of photons incident on the detector.

Measurements in units of radiance automatically take into account camera settings (for example, integration time, binning, f/stop, and field of view). As a result, images of the same subject acquired during the same session have the same signal amplitude regardless of the camera settings because the radiance on the animal surface does not change. The advantage of working with image data in radiance mode is that camera settings can be changed during an experiment without having to adjust the images or the measured ROI data. Images or ROI data can be quantitatively compared across different IVIS Imaging Systems.

Caliper Life Sciences calibrates the CCD response and lens of each IVIS® Imaging System for all the emission wavelengths. The response of the CCD is relatively flat (~10%) over the range from 500-700 nm which includes the spectral variation found in bacterial or firefly luciferase. Therefore, calibration is accurate over this range.

## Efficiency

The fluorescent signal detected from a sample depends on the amount of fluorophore present in the sample and the intensity of the incident excitation light. The excitation light incident on the sample stage is not uniform over the field of view (FOV). The profiles for all stage locations peak near the center of the FOV. The illumination intensity profile varies by up to ±30% across the entire FOV (Figure 4).



**Figure 4** Illumination profiles at different FOVs
Measurements were taken at the center of the FOV on the IVIS® Lumina Series.

Displaying fluorescent image data in terms of efficiency eliminates the variable excitation light from the measurement and enables a more quantitative comparison of fluorescent signals. When you select efficiency for the image data (Figure 2), the software normalizes the fluorescent emission image to a reference image and computes:

Efficiency = Radiance of the subject/Illumination intensity

Prior to instrument delivery, Caliper Life Sciences generates a reference image of the excitation light intensity (no emission filter) incident on a highly reflective white plate for each excitation filter at every FOV and lamp power. The data are stored in the Living Image folder.

Image efficiency data does not have units. The efficiency number for each pixel represents the fraction of fluorescent photons relative to each incident excitation photon and is typically in the range of $10^{-2}$ to $10^{-9}$. When ROI measurements are made, the total efficiency within the ROI is the efficiency per pixel integrated over the ROI area, so the resulting units of total efficiency is area or $cm^2$.

# Flat Fielding

Flat fielding refers to the uniformity of light collected across the field of view (FOV). A lens usually collects more light from the center of the FOV than at the edges. The Living Image software provides a correction algorithm to compensate for the variation in the collection efficiency of the lens. This enables uniform quantitation of ROI measurements across the entire FOV.

To apply the correction algorithm, choose the Flat Field Correction option in the Corrections/Filtering tools. The algorithm multiplies each pixel by a predetermined scale factor. The scale factor for each pixel depends on its distance from the center of the image. The scale factor near the center of the field of view is one, but can be up to two or three near the corners on the IVIS® Imaging Systems and Spectrum.

You may notice an increase in noise near the edges and corners of the FOV when flat field correction is applied– this is normal.

# Cosmic Ray Corrections

Cosmic rays are extraterrestrial high-energy particles that register a false signal on a CCD detector. Cosmic rays as well as other sources of ionizing radiation cause infrequent interactions (a few per minute) on the CCD. These interactions result in large signals that are usually isolated to a single pixel, making them easy to correct.

The Living Image software searches for isolated, high amplitude *hot pixels* and replaces them with a collective average of surrounding pixels. The Cosmic Correction option should always be selected for *in vivo* image data because hot pixels can significantly affect an ROI measurement.

Cosmic ray correction is not recommended when imaging very small objects such as individual cells. An individual cell may only light up one or two pixels and can sometimes be misinterpreted as a cosmic ray. In this case, clear the Cosmic Correction option in the Corrections/Filtering tools to avoid filtering out single-cell images.