Technical Note

In Vivo Image Analysis Options Using Image Studio™ Software

Developed for:

Pearl® Imaging System

Published February 2013. Revised November 2015. The most recent version of this document is posted at:

www.licor.com/bio/support/

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I. Introduction
A variety of image analysis options are available with Image Studio Software. Multiple measurements for a region-of-interest (ROI) are provided; however, determining which analysis method would be appropriate depends upon the nature of the animal study and questions being addressed. This document is designed to introduce some commonly used analysis methods and provide insight into how each might be implemented in a study. Examples will be provided for illustration purposes. Analyses include the Auto Shape tool found in Image Studio software, Signal, and signal-to-noise ratio (SNR) comparisons.

II. General Rules of Thumb
1) Analyze images within the same tumor model (e.g., cell lines, etc.).
2) Analyze images captured at the same resolution setting.
3) Analyze images generated at a similar focus position.
4) Image at the same time, post-injection.
5) Avoid mixing manually-drawn and Auto Shape ROIs in a single analysis. Select one or the other.
6) If using the Auto Shape tool, the standard deviation multiplier should remain constant for the complete study.

III. Auto Shape Tool
Activation of the Auto Shape Tool and identification of a couple of parameters allows boundaries of a target region to be determined automatically. By allowing the software to identify target region boundaries, the scientist is able to standardize the ROI identification process. A background region and a standard deviation multiplier are required prior to ROI determination. The background region will be used to set the baseline or threshold value for an image. The standard deviation multiplier determines the level of signal over baseline/threshold that will be included in the ROI determination.

Figure 1. A pictorial rendition of the Auto Shape analysis. A) The line graph represents the mouse image shown directly below. The red line represents a hypothetical line scan extending from the lung to the hip of a mouse. The background level was determined from the blue ROI on the hip, labeled B. The horizontal hatched lines in the graph approximate the number of background standard deviations over the background mean. The grey shaded area between the purple vertical lines represents the signal that would be analyzed if a standard deviation multiplier of 5 were chosen. B) The grey shaded area between the purple vertical lines represents a standard deviation multiplier of 10.
To explain the determination of these critical parameters, the hypothetical scan shown in Figure 1A illustrates what signal is included in an analysis after that signal has been adjusted for background. If, for example, a standard deviation multiplier of 5 is selected, the fluorescent signal present in the resulting ROI would include the portion between the purple lines extending from background to the peak (grey area). If the standard deviation multiplier is increased to 10, the ROI displayed will be very small and only include the grey area shown in Figure 1B. By increasing the standard deviation multiplier to 10, the researcher has imposed a more rigorous analysis, which may actually reduce the chance of detecting small signal changes.

**Working Example:** The first step in using the Auto Shape tool is to place a background ROI in a region that most accurately represents the tumor region of interest minus the tumor. In this example, the background ROI (white oval, Figure 2B-F) is placed on the hip opposite the tumor. The second step for the researcher is to determine the level of stringency needed to accurately screen the fluorescent signal from the tumor region. This can be dynamically reviewed in the Auto Shape tool in Image Studio Software.

The blue hatched line represented in images B-F (Figure 2) illustrates the Auto Shape tool's ROI boundaries when the standard deviation multiplier is changed from 2 to 6. Note how the ROI changed dramatically when the standard deviation multiplier reached 5. Little improvement was gained when the standard deviation was increased from 5 to 6. Notice the large section of increased fluorescence present down the backbone of the mouse. This region does run in close proximity to the tumor ROI. The Auto Shape tool is incapable of distinguishing between this background signal and that of the tumor when the stringency parameter is set too low. A more rigorous data analysis, accomplished by increasing the standard deviation multiplier, assists the researcher in eliminating signals that obviously do not represent the region of interest. In this example, tumor boundaries applied by the software when the standard deviation equals 5 represents the best fit visually to the tumor size. Once the researcher has settled on a standard deviation multiplier that best represents their data, that multiplier should be applied to the complete analysis.

### IV. Signal

**Equation:** \( \text{Signal} = [\text{Total Intensity for ROI of interest} - (\text{Background Mean Intensity} \times \text{Area (pixels) for ROI of interest})] \)

When an ROI is drawn to encompass the tumor, calculating Signal will allow comparisons of tumor size within a tumor model. These comparisons are semi-quantitative. Ideally, a regression analysis should be run to confirm that a correlation between Signal and tumor weight does exist with respect to a particular tumor model. An example presented here represents seven nude mice bearing subcutaneous prostate tumors of various sizes. Animals were injected with IRDye® 800CW 2-DG and imaged 24 h post injection. Tumors were subsequently recovered and weighed. The resulting linear regression for Signal to weight is presented in Figure 3, where 98% of
the variability in fluorescence intensity measured can be attributed to the variability in weight. For an additional example, Kovar et al. (1) demonstrates this principle with IRDye 800CW EGF in an orthotopic prostate tumor model in mice. The importance of staying within a tumor model system when interpreting data is presented in the following Working Example.

**Working Example:**
An epidermal carcinoma is implanted on the right hip and a glioblastoma on the left hip. IRDye® 800CW RGD is a targeted optical imaging agent specific to the αvβ3 integrin receptor. Note the glioblastoma tumor is ~3x smaller in size compared to the epidermoid tumor (Figure 4A). However, after administration of the probe and imaging 24 h post injection (Figure 4B), the signal for the smaller glioblastoma is greater compared to the epidermoid tumor. Why? The receptor number for the glioblastoma cell line is higher than for the epidermoid cell line and thus binds more of the optical agent. If signal comparisons were evaluated between these two tumors of differing cell origin, one would assume that the glioblastoma tumor is larger when in fact it is smaller. This is an example of why tumors of different origin should not be compared directly.

**V. Effect of ROI Size on Analyses**
The following discussion will revolve around ROI size for a tumor and the effect ROI size has on the analysis. Three ROI sizes will be screened: small, placed at the center of the targets; medium, produced with the Auto Shape tool to a specified Standard Deviation Multiplier; and large, where the target fits comfortably within the ROI. These are illustrated in Figure 5A.

Signal and SNR calculations for each tumor ROI are presented in Figure 5C. Signal is positively correlated to ROI size while SNRs are inversely correlated. In all cases, the same conclusion would be drawn for respective tumor model; however, if the limit-of-detection (LOD) for a particular mouse model is known, only values above that limit would be deemed real. For the purpose of this example, assume the LOD is a SNR >2.5. Only the small and medium ROIs would
be identified as real tumor fluorescence since their SNRs exceed 2.5 (Figure 5C). These ROIs are not diluted by the inclusion of pixels with lower signal values, resulting in higher SNRs. It would be recommended when identifying the LOD to use ROIs that do not include any added pixels which may lie outside a specific region (i.e., tumor) that could skew the study results. Ultimately, be consistent throughout image analysis when placing ROIs.

**Background ROI Size Effects on Signal Analyses:**

A more dramatic effect to the data analysis would be expected with the size and placement of the Background ROI. In this example, the ROI for the background is larger (Figure 5A) than the target tumor ROI but is placed in a similar region that closely reflects the region where we find our tumor signals. Pixels are averaged to determine the mean background signal so relative size of the ROI can differ from the tumor ROI. However, if the ROI for the background is drawn in a region that unrealistically overestimates the level of background compared to the regions where the suspect tumors are expected (Figure 5B), signal values may be uncharacteristically low. In addition, when a large uncharacteristic ROI for background is used in conjunction with a large ROI for the target, the resulting Signal analysis is low (Figure 6), increasing the likelihood a positive signal may be missed.

The reverse is also true. A background ROI placed in an uncharacteristically low signal region can cause overestimation of Signal, yielding a false positive. Consistent and careful placement of ROIs should minimize these effects.

**VI. Comparisons Between Signal, Mean SNR, and Max SNR**

Equation:

\[
\text{Mean SNR} = \frac{\text{Mean intensity for ROI of interest} - \text{Mean Background}}{\text{Standard Deviation Background}}
\]

\[
\text{Max SNR} = \frac{\text{Maximum intensity for ROI of interest} - \text{Mean Background}}{\text{Standard Deviation Background}}
\]

Not all analysis procedures are created equal. Care should be taken to select a valid analysis method for a particular study.

**Working Example:** This example addresses the use of Signal, Mean SNR, and Max SNR as measurement criteria for a competition experiment where animals representing a negative control, a dye-only control, a positive control, and a competition treatment are examined. Figure 7 contains images of the animals used for these comparisons.

Results are presented in Figure 8 for each of the analyses mentioned. The graphical analysis for Signal provides a good representation of the normalized images presented in Figure 7. The negative control animal has no measurable signal in the two tumors. Minimal tumor
signal is detected for the IRDye® 800CW carboxylate treatment. Tumors in animals receiving IRDye 800CW RGD exhibit the highest Signal intensity, while the animal receiving the blocking agent prior to the probe exhibited a reduction in probe binding and thus reduced Signal intensity.

However, when the Mean SNR and Max SNR were calculated with the same set of animals, a different conclusion might be drawn with respect to the carboxylate control tumors and their contribution to the overall understanding of the labeled agents action. With respect to the A431 tumors, data presented in the Mean and Max SNR graphs (Figure 8) seem to suggest the carboxylate dye is contributing a significant level of fluorescence (i.e., probe binding) when compared to the IRDye 800CW RGD probe. Yet, when viewing the normalized images shown in Figure 7, the images show low fluorescence in the carboxylate control animals. Why? Figure 9A and B are the same image, viewed using different intensity scales. Note the color bars for each image. It becomes clear that in fact there is retention of the carboxylate dye in the epidermoid tumor, although very low. Due to the extremely low background levels in these images, the SNRs will be high.

Unless you understand an optical agent’s action, you might interpret the carboxylate signal as an excessive amount of binding or non-specific retention in this model; however, this holds true only for the epidermoid tumor and not the glioblastoma located on the opposite hip. Is this a tumor-specific response? Notice the size of the two tumors in the white light image in Figure 7B. The glioblastoma is very small relative to the epidermoid tumor. Optical agents are known to pool, especially in necrotic regions of a tumor. Some tumor microenvironments develop elaborate vasculature to support their rapid growth. These vessels can be leaky. Either of these explanations may hold true with respect to the large epidermoid tumor. With regard to the glioblastoma, necrosis may not be present; hence, the different retention level. These data only reinforce the importance of staying within a tumor model when evaluating a particular optical imaging agent. Therefore, Signal would be the measurement criteria of choice when controls such as the carboxylate are included in the analyses.

VII. Longitudinal Series Considerations

Visual comparisons within a series of images require all images to be normalized to the same fluorescent scale or LUT; however, linking LUTs is not required for quantification. Examples would include clearance rate or optimal imaging time point identification post-injection for a particular imaging agent. The Image Studio software allows instant normalization of a set of images for quick visual screening by linking the image LUTs. In the next example, a longitudinal series will be evaluated for Signal, Mean SNR, and Max SNR.

**Working Example:** Two tumors from different origins are present on the hips of a mouse; A431 (epidermoid carcinoma) located on the right hip and U87 (glioblastoma) on the left. In the case of an image series such as this, the initial image post-injection (taken minutes after the injection; Figure 10A) can be extremely high in fluorescent signal. The Auto Shape tool will not be able to detect any tumor boundaries because the tumor signal is indistinguishable from background. This is a case where manual and Auto Shape tool ROIs may be required to tell the complete story. Hence, ROIs for the tumors can be placed by using the white light image for localization in the very early images and copied to the fluorescent images. The resulting tumor SNR will be very low at this point in the series. The data from Figure 10A was not used in subsequent analyses but does illustrate the low SNR when background levels are high.

All images have been normalized to a common LUT for visual comparison. Data analyses from the ROIs placed with the Auto Shape tool in Figure 10B-H are presented in Figure 11. Again, the Signal data best represents what we visualize in the images with a steady decrease in Signal intensities over time.
When Signal is used to identify the optimal imaging time point post-injection, the data would suggest the optimal time point should be 4 - 8 h post-injection (Figure 11) when signals are high. However, if the measurement criteria is a SNR calculation, one might conclude the time point may be closer to 48 h post-injection when values have stabilized. While Signal yields an estimate of the overall signal intensity for a region corrected for background, the SNRs will give the researcher an assessment of overall quality of the signals provided in an image between tumor and background regions. It is generally preferred to analyze signal that is significantly higher than the background. The greater the difference between tumor and background signals, the more confident the researcher can be with the resulting data analysis.

VIII. Sensitivity With Respect to Time

How is Signal changing over time with respect to a particular tumor model and optical agent? This data may be helpful in identifying time points when the Signals are rapidly changing or remaining stable.

Equation: \[ \text{Sensitivity} = \frac{\text{Signal}}{t} = \frac{\Delta \text{Signal}}{\Delta t} \]

where \( t \) = time and \( \Delta \) = change.

How does Signal change as a reflection of time? Sensitivity of Signal with respect to time was reviewed by calculating the slope of the lines between points in the longitudinal Signal data shown in Figure 11. The slopes for lines before and after each individual point were averaged to reflect the slope for a hypothetical line running through said point. This was then adjusted for the point’s Signal. That data is presented in Figure 12.

If we model Signal with an exponential decay, then:

\[ \text{Signal} = \text{Signal}_0 e^{-\frac{t}{\tau}} \]

where \( t \) is time, \( \tau \) is the time constant, and \( \text{Signal}_0 \) = signal at time 0.

For this model, the sensitivity of the Signal with respect to time is constant, \( \frac{1}{\tau} \).
The panel inserted in Figure 12 shows the flattening of the curves for both tumor models once the signal sensitivity is adjusted with respect to time. A flat response suggests that there is no “optimal” point for imaging from the standpoint of time. This would effectively allow the researcher more freedom in selecting a time point for imaging that suits their situation; however, once a time period for imaging is chosen, it should be used for the duration of the study.

Therefore, use of multiple analyses for a longitudinal series of images will help the researcher identify an optimal imaging time point that meets their experimental needs. Under the experimental conditions used in our example, Signal does change over time; however, the sensitivity of the Signal with respect to time is fairly constant, ultimately providing flexibility in the time point selection. Signal and SNR determinations provide additional clues in identifying an imaging time point that balances image signal intensities between tumor and background. With respect to this example, the peak SNR appears to have been reached by 24 - 48 h, and Signal has dropped approximately 30% by 24 h but almost 75% by 48 h. With these data in hand, it would seem prudent to image at 24 h when 70% of the Signal is available and SNR is relatively high.

IX. Summary
We have attempted to demonstrate some of the more common methods of data analysis currently being used in in vivo imaging with 2D imaging instrumentation. We have provided examples of animal studies using the Pearl Imaging System and Image Studio Software to illustrate the importance of using a proper method of analysis, as well as how some practices in image analysis may actually alter an end result. By understanding the intricacies of data analysis with respect to animal imaging, hopefully some pitfalls can be avoided. Certainly we have not exhausted the many options for data analysis here, but hope to have provided the researcher with a tool to help with the transition to a new imaging modality.

X. References