TECHNICAL NOTE

General and technical considerations for background subtraction in 2D fluorescence using IVIS imaging systems.

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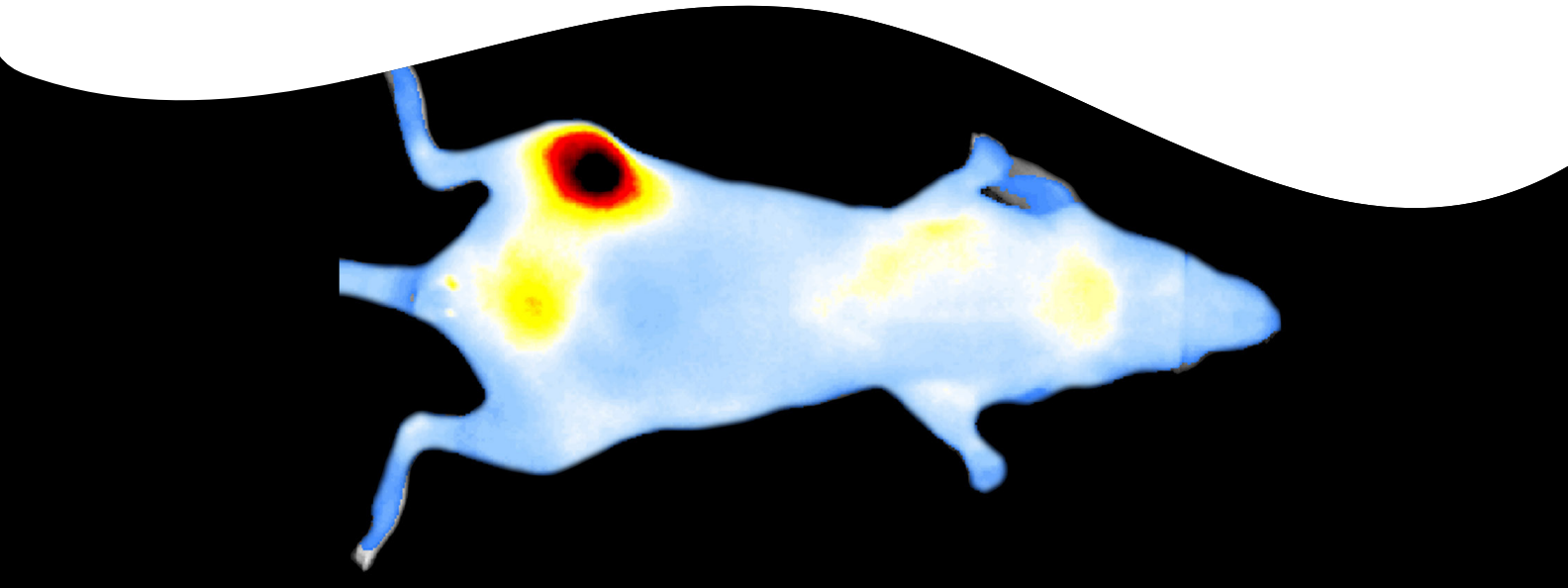
Quantitative *in vivo* optical imaging can provide information at the cellular biomarker level regarding disease states and therapeutic response, and Revvity's bioluminescence and fluorescence imaging reagents and IVIS® imaging systems provide powerful tools to facilitate this research. Bioluminescence imaging presents fewer challenges with regard to analysis procedures and quantification due to the selective localization of signal in regions associated with genetically-expressed luciferase. Fluorescence imaging offers a different approach to small animal research, utilizing near infrared fluorescent imaging probes specific for a wide variety of biomarkers for disease- or therapy-related biological changes. However, fluorescence imaging requires strategies to accurately compensate for mechanistic or non-mechanistic fluorescence background; signal is derived from systemic injection of these probes which will generate target signal as well as signal in sites of probe metabolism or non-target sites of mechanistic biomarker expression. Without proper fluorescence background comparison or subtraction, results may underestimate biological changes or the magnitude of therapeutic efficacy, whereas excessive background compensation can reduce sensitivity in the detection of lower intensity fluorescence changes. By careful analysis of epifluorescence (2D) imaging datasets, objective independent measures can be compared to fluorescence quantification to assure that appropriate background fluorescence is identified and removed. As background signal can vary depending on the disease model, anatomical site, and imaging probe used, we used a variety of preclinical imaging datasets and included several imaging probes with different clearance and biodistribution profiles.



Sources of fluorescent background

Optical imaging has emerged over the past twenty years as a powerful modality, showing strong utility in non-invasive, highly-sensitive imaging in preclinical small animal research. For proper quantification, it is important to understand the sources of wanted and unwanted signal.

Bioluminescence imaging (BLI): Background signal is not an issue. BLI is unique in that it offers a simple and robust imaging approach with little or no background with which to contend (see Figure 6 A&C). BLI signal is generated by luciferase/luciferin interaction rather than by an excitation light source, eliminating the excitation of a variety of tissue molecules with the potential for fluorescence.

Fluorescence imaging (FLI): Multiple sources of autofluorescence and background can interfere with imaging. Near infrared (NIR) fluorescence imaging (FLI) is an alternative mode of optical imaging that utilizes exogenously administered fluorescent probes, offering a broad capability in gaining specific biological insight. A light source is used to excite probes within the tissue, and at some wavelengths this can also excite biomolecules within the tissue itself, yielding interfering background signal.

Somewhat higher levels of tissue autofluorescence can limit the sensitivity of detection of exogenous fluorescent probes, particularly in the visible light wavelength range from 400 to 650 nm.

Additionally, the chlorophyll component of mouse chow can also contribute background signal (640 to 700 nm) within the stomach and intestines that can significantly interfere with imaging in the far red and near infrared (NIR) fluorescent regions.

When using NIR fluorescent imaging probes, it is important to avoid the use of common mouse chows and switch to chlorophyll-free (i.e. alfalfa-free) chow. If this is done, the most important contributor to background fluorescence is generally from the imaging probes themselves and their distribution and clearance within experimental animals (see Figure 6 B&D). Some probes are dominated by renal clearance, generating considerable background signal in kidneys and bladder, whereas others show predominant hepatic clearance, generating background signal in the liver region. Depending on the region of the anatomy to be imaged (i.e. whether the imaging site is near clearance tissues) and the time point (i.e. the time relative to tissue clearance) this probe clearance-related background may or may not be of much importance.

All of the contributors to background signal need to be considered when developing/adapting a mouse model for imaging readouts, for example selecting sites for tumor placement or inflammatory stimulus that are distal from known sites of background interference.

Table 1: Sources of Background Fluorescence.

	Background signal	BL	VIS	NIR
Imaging Process	General increased signal within the imaging field, due to background light, system noise, inherent phosphorescence of plastics and other materials placed in the imaging field. This is greatly minimized in Revvity's imaging systems.	Very Low	**Yes**	Very Low
Autofluorescence and Autoluminescence	There are many molecules present in living tissue that can be biochemical sources of autofluorescence, including tryptophan, NADH, collagen, elastin, flavins, and prophyrins. Main excitation peaks are in the visible part of the spectrum.	Very Low	**Yes**	No
	Autoluminescence can arise from endogenous cellular oxidative reactions which can generate light-emitting molecules such as triplet carbonyls and singlet oxygen. This signal is extremely low and of no practical concern for bioluminescence (BL) imaging.			
Chow Fluorescence	Regular rodent diets generally contain large amounts of alfalfa, and therefore chlorophyll, which fluoresces in visible (red) to NIR light wavelengths. Alfalfa free chow should be used to minimize background fluorescence.	No	**Yes**	**Yes**

Table 1: Sources of Background Fluorescence (Continued)

	Background signal	BL	VIS	NIR
Fluorescent Probe Clearance	Non-mechanistic background signal (i.e. signal not related to specific targeting of the probe) occurs due to the circulation and clearance of probes. Smaller molecular weight probes may show higher skin, kidney, and bladder signal, whereas larger probes may show high clearance signal in the liver or intestines.	N/A	Yes	Yes
Mechanistic Localization of Probes in Non-Target Sites	Unwanted retention of probe signal can also occur in a mechanistic fashion (i.e. localization outside of the desired imaging site in a region also expressing the molecular target). This includes localization in skin lesions and nicks from depilation.	N/A	Yes	Yes
Technical Issues During Animal Handling	Effective epifluorescence imaging requires removal of hair, which scatters and blocks FLI. Accidental skin nicks/cuts during shaving, or skin irritation due to prolonged depilatory cream exposure, can increase fluorescent probe localization to these affected areas.	N/A	Yes	Yes

BL, bioluminescence; VIS, visible fluorescence; NIR, near infrared fluorescence

Reasons to consider correcting for background fluorescence

When analyzing fluorescence imaging data (even without taking fluorescent background into account) the results are valid and will provide robust and statistically significant data. However, although fluorescent imaging probes can provide excellent targeting of disease biology, they also distribute widely throughout the body and can vary in routes of clearance and kinetics of background interference. In some cases (depending on the intensity of the signal at the target) more that 50% of your signal in the target region could be attributed to background contribution. This means that some sort of objective and consistent approach for correcting data for background contribution is needed for proper interpretation.

Taking background into account in your analysis will provide higher precision and improved data interpretation. This is particularly important when making other calculations, such as % inhibition, background, or ratios of two different imaging probes. Consider Figure 1 below, hypothetical datasets in which there are untreated animals and treated animals but the examples differ only in background levels. Assuming there is no background yields a calculation of 44% inhibition (Example 1), however Examples 2 and 3 show the impact of a modest background and a high background, with percent inhibition raising to 57% and 80% respectively.

Background correction facilitates accurate multiplex comparison and ratio analysis. The lower panel, shows a hypothetical example of a multiplex fluorescent imaging study using two different probes (on 2 separate channels). The same principles apply as in the upper panel, however this figure shows that actual adjustment of the data by background subtraction may help to graph the data more clearly (center). In addition, the assessment of the biological profile changes in the tumor, by examining ratios of probe 1 and probe 2, is clearer when taking into account the sometimes dramatic background differences between fluorescence channels and/or different probes.

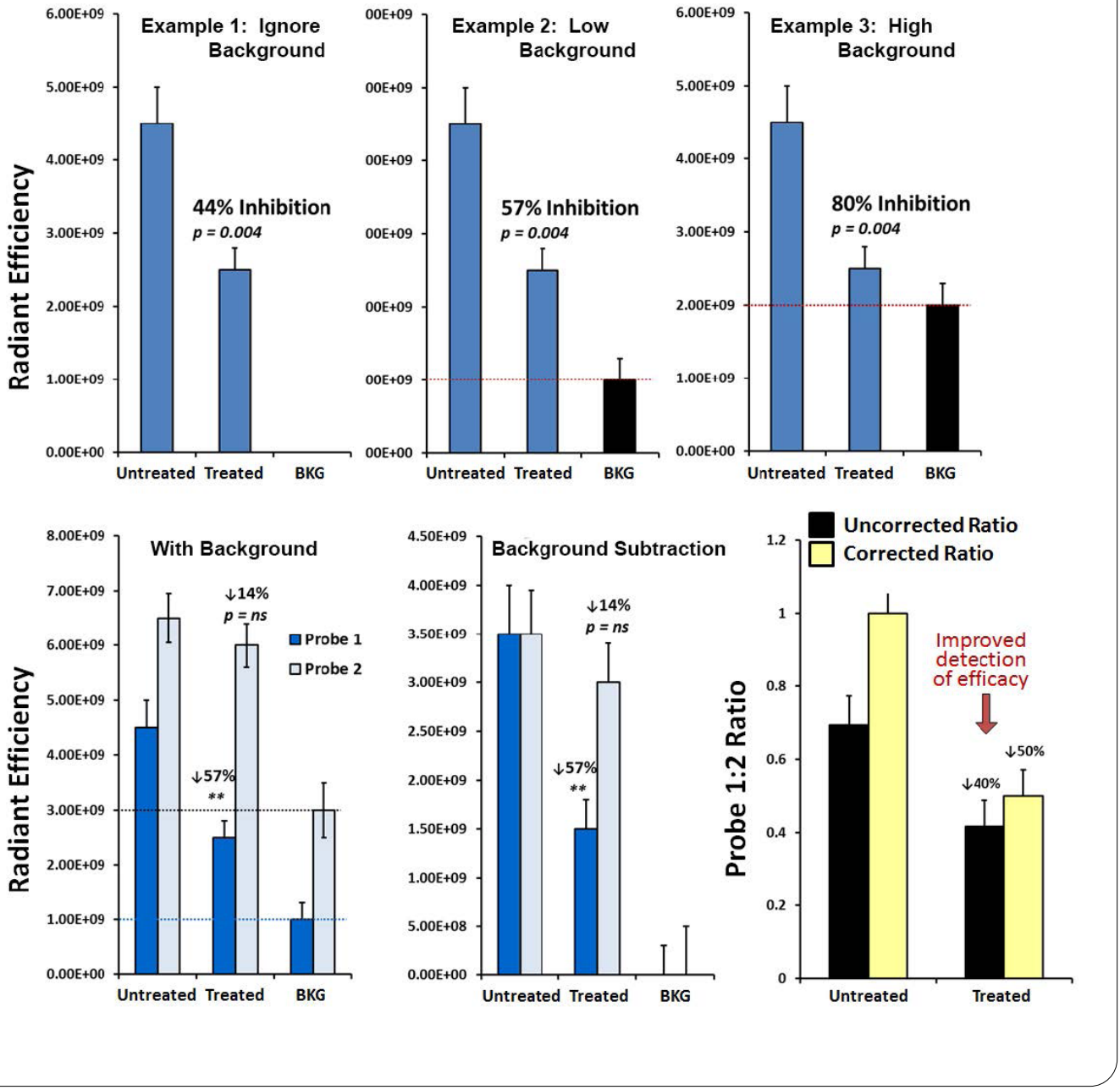


Figure 1: **Theoretical data illustrating the impact and importance of proper background subtraction.** Theoretical tumor data (upper panel) in which a treated group shows lower signal than an untreated group. Background is ignored (left), defined as modest (center), or high (right). Theoretical data using two probes (lower panel) in which different signal is generated in response to treatment. Data is graphed without correcting for background (left), correcting for background (center), or it is graphed as corrected or uncorrected ratios of the two probes (right).

Study design and background fluorescence correction strategies

When you design your imaging study, it is generally useful to include negative control animals that receive injection of probe(s), providing a true baseline for background probe signal. However many researchers prefer to determine background within their diseased animals, for example using a contralateral site for background analysis.

Depending on your study design, there are three basic types of background correction; 1) using an internal contralateral background Region of Interest [ROI], 2) using an internal non-contralateral background ROI, and 3) using an ROI identical in size and placement in control, probe-injected mice.

The first two approaches are commonly used in cancer research in which researchers are generally comparing tumor bearing animals undergoing vehicle versus drug treatment. As the disease itself can affect the animals' basic physiology, it often makes the most sense to assess background signal in the same animals. Please note, however, that the choice of contralateral or other control ROI sites in some cases may be impacted by the underlying lack of bilateral symmetry (e.g. torso regions). The third study design scenario, using probe-injected controls, is used in some cancer studies, and mostly in disease induction models (like arthritis).

The first two approaches work well with Living Image® software automatic background subtraction, where each mouse is its own control. It is recommended to pursue this approach unless it is not possible. The third approach requires manual subtraction of mean background signal from all control mice for better accuracy, necessitating manual ROI size correction (if needed) and manual subtraction (See Figure 5).

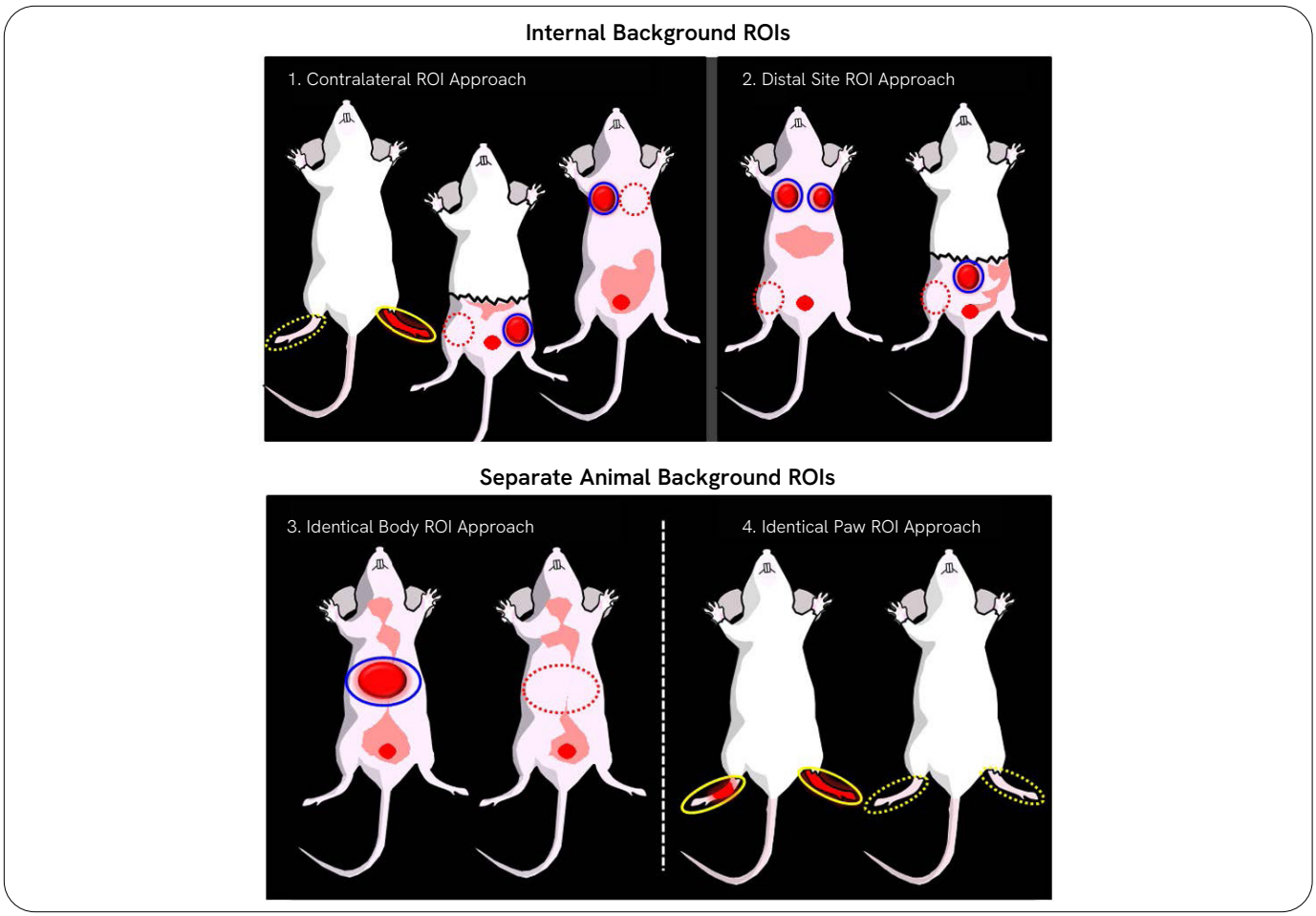


Figure 2: **Illustration of background correction strategies**. Depending on the particular animal model, the presence or absence of hair, and the target region of interest, there are different strategies suitable for applying to background fluorescence subtraction. These approaches include the use of contralateral sites, distal sites, or identical sites in control animals.

Living Image software: measurement ROI generation and placement

In order for optical imaging to be of use in preclinical research, quantitation is very important, and clear tools and strategies for analysis must be available. Living Image software provides ROI tools (in the Tool Palette) that allow you to specifically select and analyze regions of fluorescence and/or bioluminescence signal. You can use the circle or square ROIs to simply capture and quantify region(s) containing signal, or you can use the free draw or auto ROI approach to closely size ROIs to the biology (Figure 3A). Just use the drop-down menu to first select "measurement ROI" and then choose the ROI type and use the drop-down menu for that ROI type to select how many ROIs to place. Auto ROIs require setting a slide threshold to define the boundaries of the ROI, with higher threshold (%) decreasing the ROI size. A "right-click" on the displayed ROI on the image further allows you to edit ROI properties like line thickness, color, dimensions, and label (not shown). Select "measure ROIs" to open a table with your data. This example shows data not yet corrected for background contribution (Figure 3B).

The choice of ROI type, placement, and sizing can all have a significant effect on signal measurement (see Figure 6). This will be further discussed and some guidance will be offered with regard to ROI strategies.

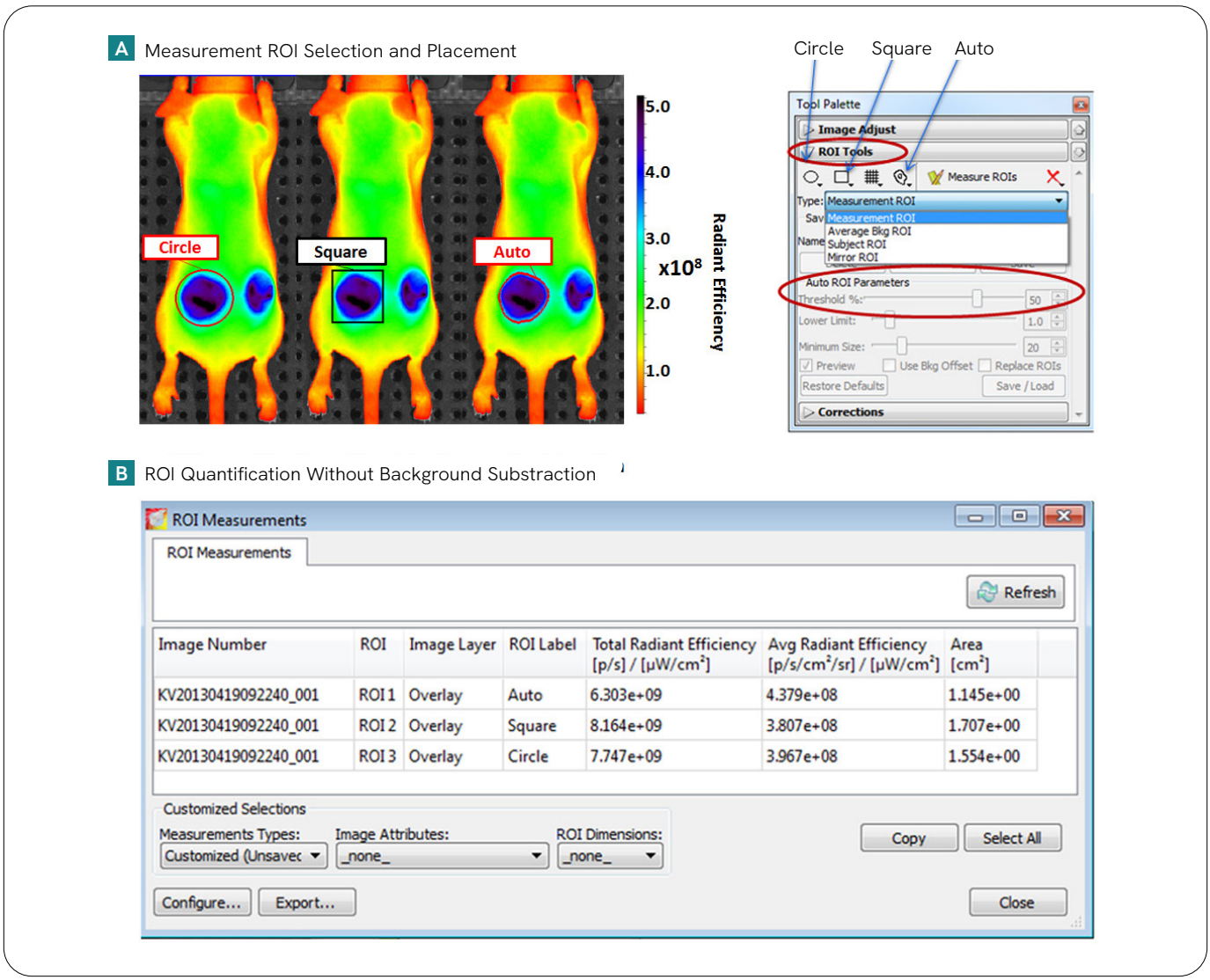


Figure 3: **Measurement ROI placement.** (A) The Tool Palette offers four different options for ROI selection and placement, and sizing /re-sizing is easily performed using the computer mouse. (B) A simple click of the "Measure ROIs" button generates a table of data, and the output can be configured to include a number of acquisition, quantitation, and ROI parameters.

Living Image software: Background ROI placement

The Living Image software Tool Palette (Figure 4A) also allows you to establish and place background ROIs for appropriate measurement correction.

1. Use the "Type" drop-down menu to first select "Average Bkg ROI".
2. Choose the ROI shape (only circle or square are options). Use the drop-down menu to select the number of ROIs to place.
3. Resize and move background ROIs as appropriate.

Background ROIs will be displayed with a dotted line shape. The precise size/shape of background ROI created is not important (correction is applied), but placement should be in an appropriate location and sized large enough to get a good regional average (see Figure 7).

Automatic background subtraction provides easy data correction. Placement of internally-placed background ROIs offers the useful approach of using each animal as its own background control for data correction. This automated approach adjusts data from each background ROI based on the ROI size prior to performing the subtraction. A "right-click" on each of the displayed measurement ROIs opens a drop-down menu that allows you to link each ROI to the appropriate background ROI (not shown), generating automatic background correction.

Measurement of fluorescent signal (Figure 4B) shows that background correction can have a significant impact on quantification, in this case subtracting 42-48% of the total signal. However, background ROI placement can be somewhat subjective, so the next few sections provide guidance on the utility and strategies of proper background subtraction.

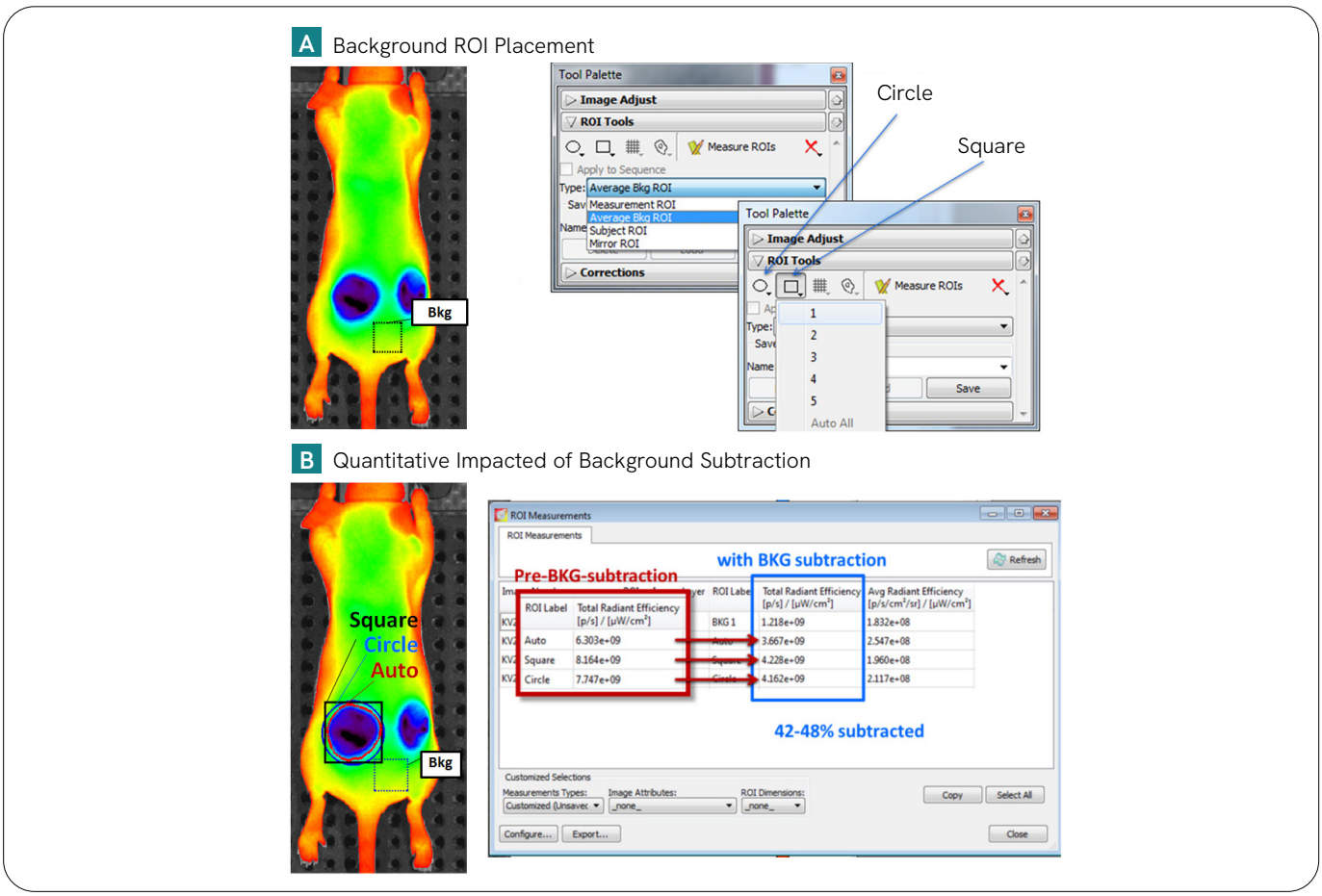


Figure 4: **Background ROI placement.** (A) The Tool Palette offers two different options for background ROI selection (circle and square), and sizing /re-sizing is easily performed using the computer mouse. (B) A simple click of the "Measure ROIs" button generates a table of data that automatically subtracts background (corrected for ROI size differences) from each linked measurement ROI. Data output can be configured to include a number of acquisition, quantitation, and ROI parameters.

Manual calculation approach to fluorescence background subtraction

Manual background subtraction is best when background ROIs are placed in separate animals. Manual background subtraction is needed if you are using background ROIs in separate control mice, because you will likely want to use the mean of multiple control mouse background values to correct data generated from the target site(s) for each individual mouse. This is a simple approach, using data export to a spreadsheet, and it is illustrated below in Figure 5A/B.

For manual subtraction:

1. Place appropriate measurement ROIs at site(s) of interest in each tumor mouse. These may need to tailored in size to capture the signal/biology.
2. Place additional measurement ROIs *(you don't need to use "background ROI")* in the same anatomical region in control mice (using tools described in Figure 4).
3. Please note that calculations are greatly simplified by using ROIs of the same size for all background site ROIs, and it is not important to accurately match them to the sizes of target site ROIs.
4. Select "measure ROIs" on the Tool Palette, and use the "configure" button to set the data output to include Total Radiant Efficiency (Total RE) and Area (cm^2).
5. Export or copy/paste data to a spreadsheet for analysis. You will need to determine the mean of the Total Radiant Efficiency of your background ROI values and the mean background ROI Area in cm^2.

Figure 5B shows the calculations for subtracting background ROIs that are size mis-matched to the target ROI size. (background values are correct to the size of each tumor ROI.)

6. Proper adjustment of the mean background Total RE can be achieved by multiplying it by Tumor ROI size (cm^2) and dividing by background ROI size.
7. The adjusted mean background total RE is then be subtracted from that particular Tumor total RE.
8. Repeat calculations 6 and 7 for each Tumor ROI (which will likely differ in size from the other Tumor ROIs).

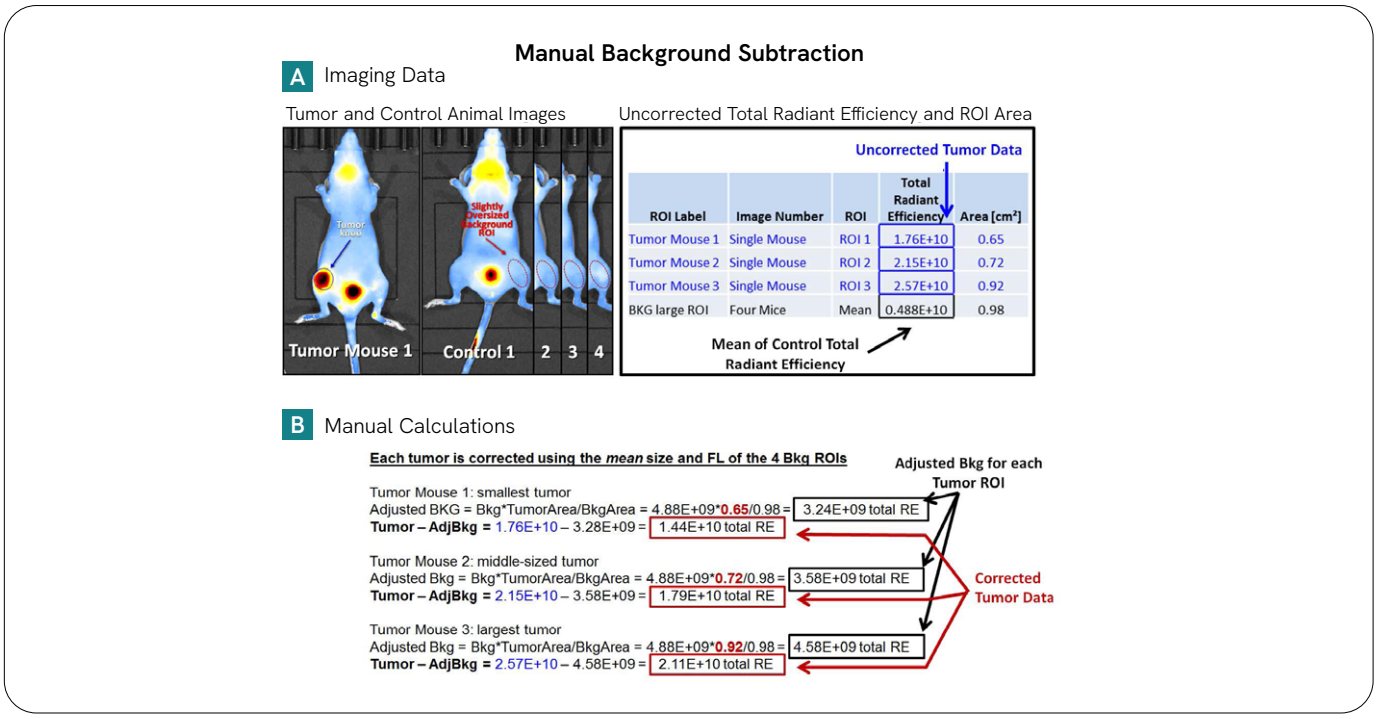


Figure 5: **Manual Bkg subtraction.** Living Image quantitative data can be exported to a spreadsheet for manual analysis. This is useful for instances in which control mouse data is not collected in the same dataset sequence or when performing background corrections based on averages of control data. Upper panel shows a single representative tumor mouse and four control mice (left) and a table of Tumor ROI data for three mice as compared to the mean data for four control mice. The lower panel shows how to use size-corrected background ROI values to correct for background signal contribution.

Fluorescence biodistribution and the impact of ROI sizing

Previous pages have described the rationale and approaches for fluorescence background subtraction. The remaining sections of this technical note will provide additional information regarding proper Measurement/background ROI sizing, background ROI placement, and ways to understand if you are correctly determining FLI background.

So far, fluorescence has been discussed, but it is useful to compare and contrast the two major modes of optical imaging (BLI and FLI) that differ significantly in how signal is generated. BLI signal can only arise where luciferase is expressed (generally engineered tumor cells or bacterial lines) and signal is generated by luciferin/luciferase interaction. Greater than 95% of the signal in the mouse is constrained to the target site (i.e. the site at which luciferase-expressing cells are present), and there is little or no signal outside of the target site (see Figure 6A below).

Of more concern is FLI, which uses systemically administered probes that bear fluorescent tags. These drug-like probes generate complex biodistribution patterns throughout the animal, with enriched accumulation at the target site. As expected, an example NIR FLI probe shown below (Transferrin-*Vivo*) demonstrates a broad distribution of fluorescence throughout the body (~85%) with approximately 15% of the signal retained at the target site (see Figure 6B below).

Control probes, i.e. probes not specific for the site of disease (like IVISense™ Osteo probe below), deliver less that 1% of fluorescent signal to the target. *(Note: these 2D numbers underestimate deep tissue non-target signal, so in reality the absolute percentage of most targeted imaging agents getting to the target is ~2-8%.)*

BLI Analysis: Larger ROIs are better, no background correction. To assess the impact of tumor region of interest (ROI) size on tumor fluorescence quantification, a variety of sizes of ROI, from undersized to oversized, were used (see Figure 6C). BLI analysis is very simple, with the dominant target signal allowing a lot of flexibility in ROI sizing; the ROI can be sized substantially larger than the target biology without compromising quantification. Based on these performance characteristics, BLI requires no application of background assessment for optimal interpretation of imaging results. There is little or no background to interfere with specific imaging.

FLI Analysis: Minimal ROIs are better, need background correction. FLI analysis requires careful ROI sizing to the target biology, as the lower, but significant, non-target related signal outside the target can complicate quantification with oversized ROIs (see Figure 6D). This means that properly sized ROIs generally will maximize the target: background ratio and provide greater consistency in analysis, minimizing a potential source of variability in your study.

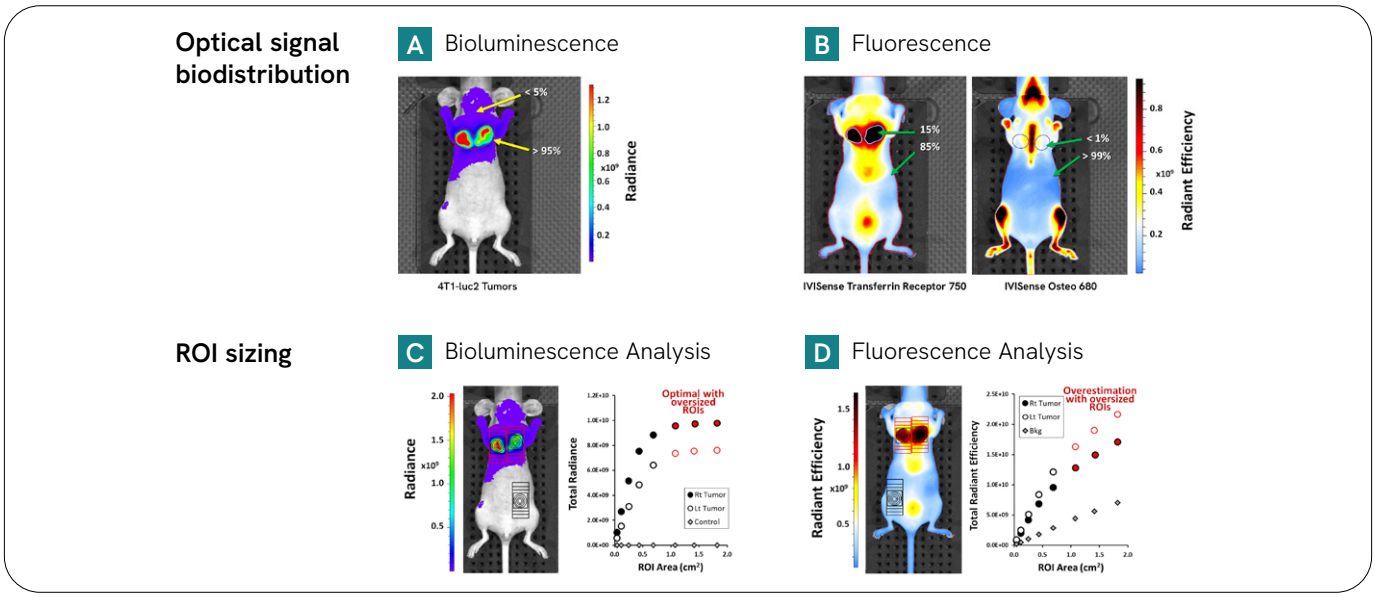


Figure 6: **BLI and FLI tissue distribution.** (A) more than 90% of 2D emission light from bioluminescent tumors is in the tumors themselves, whereas (B) approximately 15% of 2D fluorescence emission from a targeted probe comes from the tumor. (C) A range of sizes of ROIs reveals that ROIs for 2D BLI analysis can be as large or larger than the target. (D) In contrast, ROIs for FLI need to be appropriately sized to the target or significant background signal will be captured.

Characterizing background ROI variability

Now that we understand the proper way to capture target site fluorescence signal, it is important to fully understand background fluorescence. A number of factors must be considered when choosing specific sites for placement of discrete background ROIs. Where you place your background ROI can have a tremendous effect on quantification and interpretation of your fluorescence data.

Background ROI placement, if not done carefully, can be a significant source of variability in your analysis. Figure 7A illustrates the range of possible background results from which to choose as compared to the choice of a low signal region in the flank of the animal. Note that the distributed ROIs are all set to approximately the average of the two tumor ROI sizes for the purpose of comparing total signal (which will be dependent on ROI size).

This particular dataset shows approximately a two-fold range of background intensity, from the minimal flank signal to higher neck region signal. Even the low flank region signal is approximately 25-40% of the uncorrected tumor signal.

Background ROI size should be similar to the size of the target ROI to minize sampling bias. Figure 7B illustrates the use of differently-sized background ROIs constrained to a pre-defined flank region (n=4 for each ROI size). Very small ROIs *(although corrected for size difference prior to subtraction)*, even when confined to a small anatomical region, show high variability due to sampling bias of heterogeneous background signal. This bias can be minimized by using background ROIs sized similarly to the tumor ROIs. Properly sized and oversized ROIs are generally more forgiving of small differences in background ROI placement.

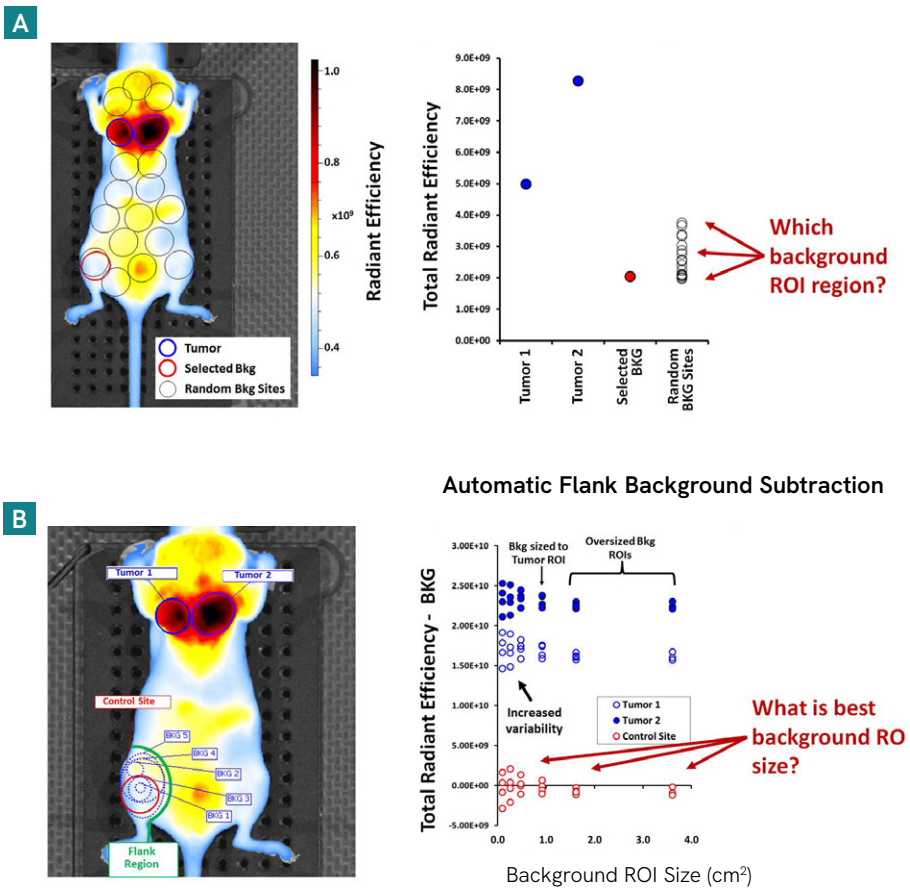


Figure 7: **Background ROI size and location impact on variability in measurement correction.** (A) A 4T1 tumor-bearing mouse was imaged by IVISense Bombesin Receptor 680 (2D FLI) and assessed for the range of background signal throughout the body. (B) The potential for variability and sampling bias, even within a smaller selected flank region, was assessed by placing multiple ROIs of different sizes throughout the flank.

Placement of non-contralateral background ROIs considering the impact of background variability

Once background heterogeneity and the impact of ROI sizing is understood, it is important to determine objectively the right amount of background subtraction to apply. Is there a general and objective strategy that can be applied to any fluorescence imaging dataset? Such a strategy should offer a consistent approach to ROI placement and sizing with resulting background correction minimally affecting the real fluorescence signal (i.e. both in target as well as sites of clearance and off-target mechanistic accumulation). This further assures that other dimmer sites of potential biological interest (e.g. smaller sites of disease) will not be missed.

Living Image (v4.5) line profile tool can help to illustrate the dynamic range of target and background signal. To more quantitatively understand the heterogeneity of target and non-target signal intensities across the body, Living Image (v4.5) offers a "Line Profile" tool *(see Tool Palette/Image Adjust/Image Data/Line Profile in Living Image software when analyzing a dataset)*. A free-hand profile line can be placed across the body of the subject to capture a graphical representation of signal. Figure 8A shows 4T1 tumor bioluminescence signal and the line profile quantification (blue solid line), indicating tumor signal and essentially no background. In contrast the same line profile approach for three different fluorescence probes (Figure 8 BCD, red lines) shows complex tumor peaks with additional fluorescence hot spots in various regions depending on the probe.

Background ROI placement at a distal, low-signal site can offer an effective option to avoid over-correction. As a frame of reference for understanding the FLI background in these animals, two simple approaches for assigning background were attempted; 1) distal flank background ROI placement and 2) a tumor-proximal ROI to capture the elevations right next to the tumors. The average signal for each approach (i.e. a measure of average pixel "brightness") is represented on each Line Profile chart. This graphical representation allows you to better understand the impact of using different background levels for data correction.

In all three probes, the tumor proximal background values were higher than much of the non-target clearance signal, suggesting that this approach would lead to over-correction. Flank backgrounds were much lower and preserved the majority of the heterogeneous tissue clearance signal. For reference, the BLI Line Profile (green dotted line in BCD) was superimposed on the fluorescence Line Profile, setting the base of the line on the average flank fluorescence background cut-off. The yellow region indicates the theoretical quantified portion of the signal, taking into account the ROI determination of tumor margins and the background subtraction (indicated in gray).

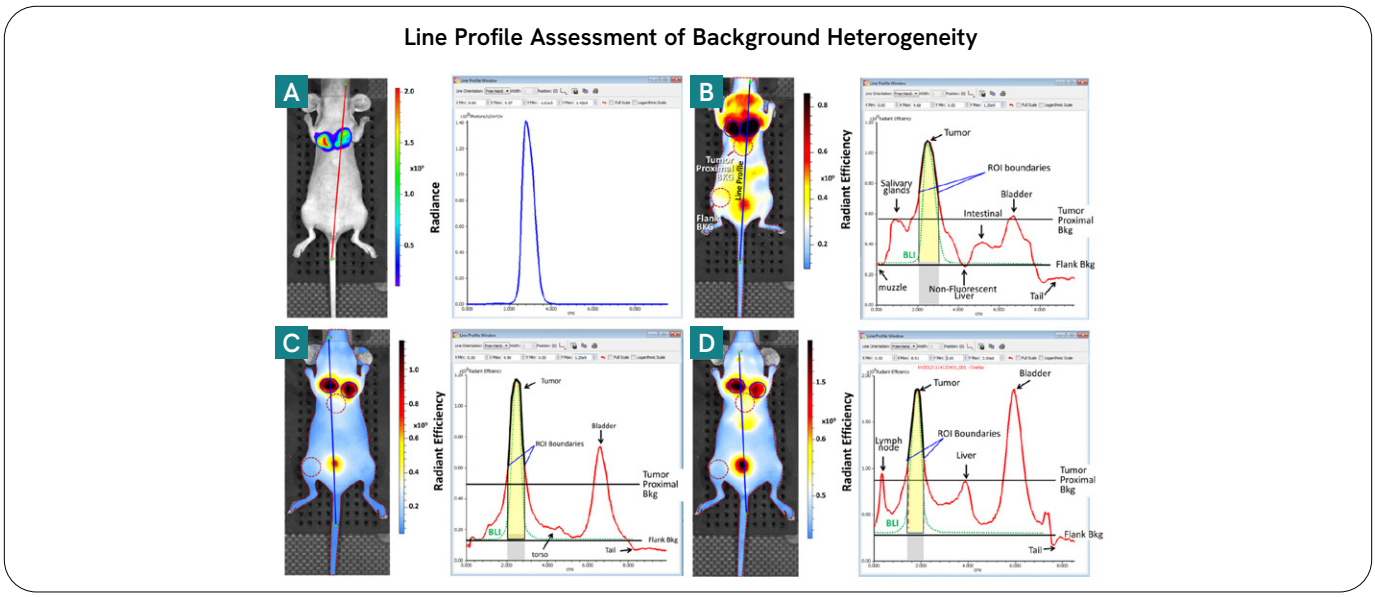


Figure 8: **BLI and FLI imaging backgrounds defined by Line Profile.** 4T1 tumor bearing mice imaged by BLI (A), IVISense Bombesin Receptor 680 (B), IVISense Pan Cathepsin 750 (C), and IVISense MMP 680 (D), using the Living Image "Line Profile" tool to illustrate probe-dependent background differences. For reference, background ROIs were placed proximal to the tumor or in the flank.

Identification of alternative distal site background ROI options

Closer examination of the mice shown in Figure 8 allows careful identification of all of those regions that could possibly be suitable as background regions based on results from the line profile tool (Figure 9 ABC). Obviously, this identification is affected greatly by the position of the target site, forcing the exclusion of upper thoracic ROIs from consideration in these particular examples.

A variety of sites can often be identified for background assessment, offering user flexibility. Although flank background ROIs often work well, it is not always possible to use this approach. Alternatively, once you have identified an approximate background range, a few different regions can be identified in each mouse that could be suitable for background ROI placement. Other lower torso regions are comparable in fluorescence levels to the flank, but this depends on the specific probe(s) being imaged. For IVISense Bombesin Receptor probe, the background placement options are somewhat limited, however the other two probes offer a number of background site possibilities. Whatever site is selected, it is important to be as consistent as possible.

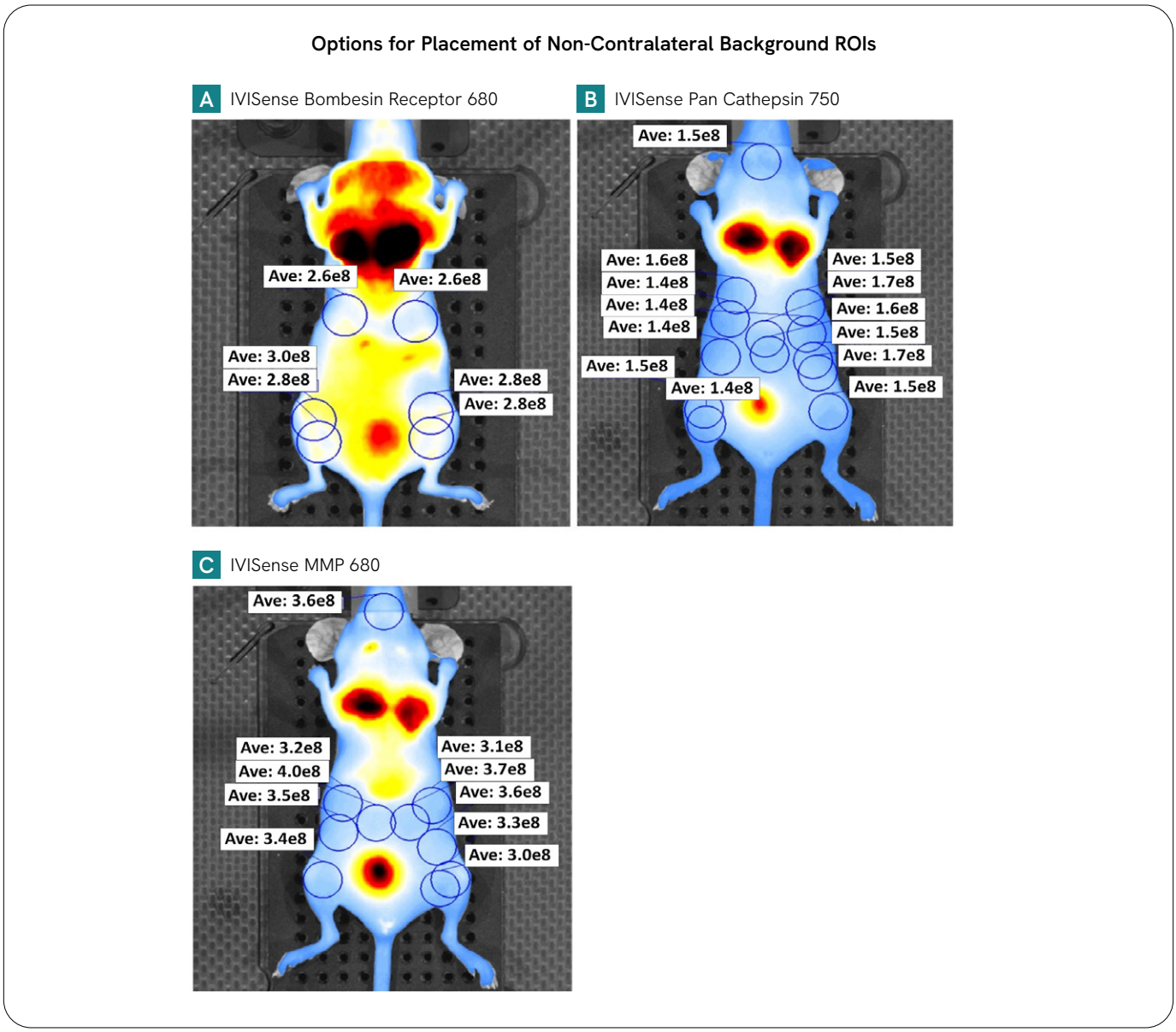


Figure 9: **Identification of possible background ROI positions.** 4T1 tumor bearing mice imaged using IVISense Bombesin Receptor 680 (A), IVISense Pan Cathepsin 750 (B), and IVISense MMP 680 (C), with placement of multiple background ROIs in regions that agree with background average signal determine in Figure 7. Any ROIs that were not within 15% of the flank value were deleted from the image.

Comparing internal and control animal background subtraction approaches

Both contralateral and control animal background approaches can yield very similar results. The different background subtraction approaches described in Figure 2, internal and control animal use of discrete background ROIs, are both viable options and can be selected based on your own unique animal model needs. To illustrate this, the same flank tumor model was used to quantitatively assess these subtraction approaches as illustrated below. Three ROIs were placed, 1) tumor ROI, 2) contralateral ROI, and 3) background ROI, for each approach.

These two background subtraction approaches focus on matched anatomical regions for all ROIs, and the results show that you can generate very similar quantitative results. More importantly, applying a consistent approach to background correction will improve the accuracy of quantification and help in appropriate interpretation. Discrete smaller background ROIs may require extra care to be consistent, whereas larger ROIs may be more consistent but harder to avoid background signal from bladder or other sites of clearance.

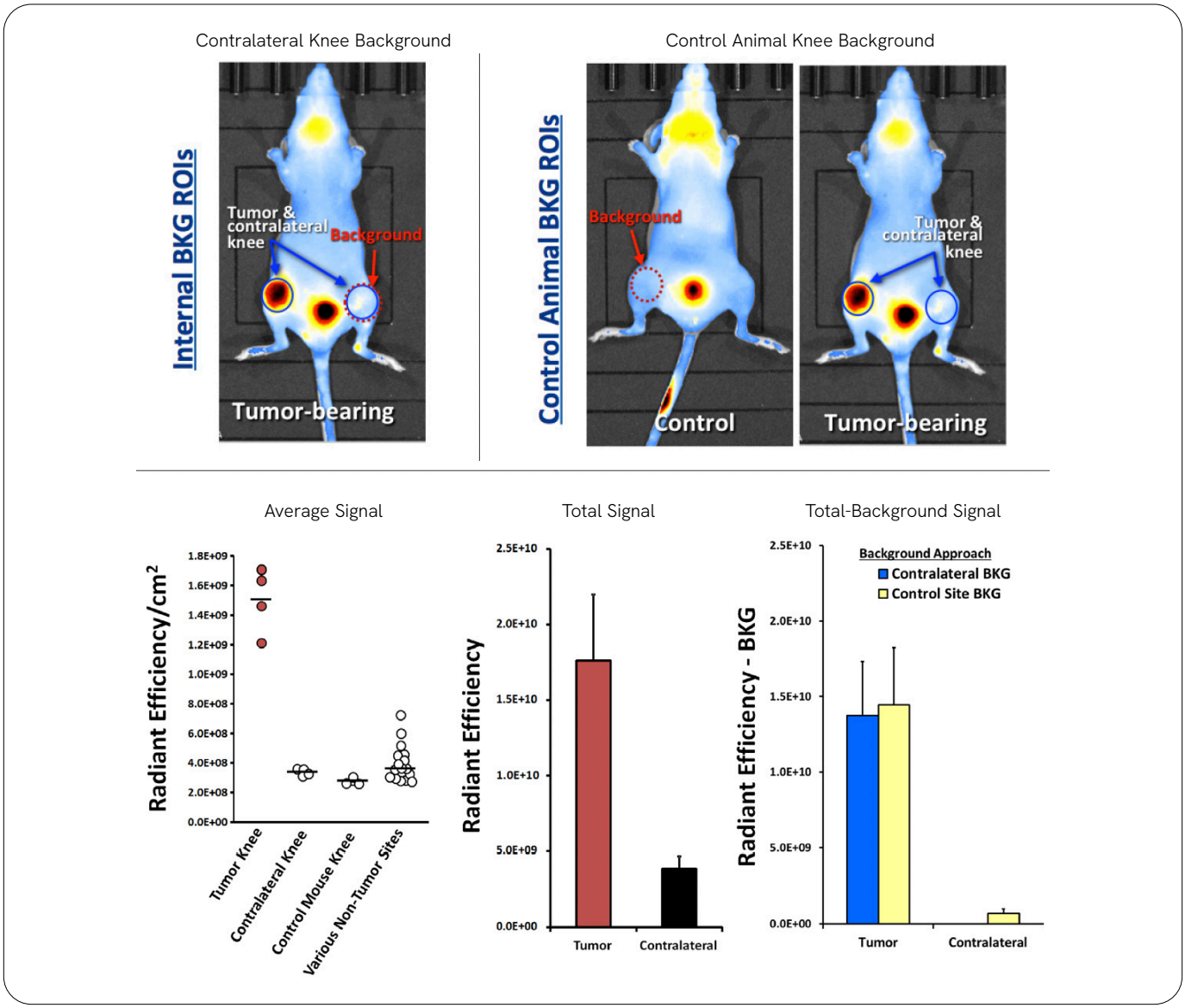


Figure 10: **The different background subtraction approaches applied to a flank tumor model.** The upper panel of images illustrate the two internal background ROI approaches. The lower panel of images show the ROI quantification with and without the two background subtraction approaches. Both approaches yielded comparable results, but the internal background subtraction was automatic and the control background subtraction was calculated manually (size normalization of total background values followed by subtraction of total from background Tumor values).

Better quantification with background subtraction: Cross-validation to BLI

To visualize the impact of background subtraction on fluorescence quantification (Figure 11A), tumor-bearing mice (bearing different-sized tumors) were assessed for both tumor and background signal intensity (average fluorescence, red line). A flank background ROI was examined, and it captured average signal intensity consistent with a reasonable background level as compared to the body line profile (Figure 11B).

Background correction was applied to fluorescence datasets for each individual mouse using corrected flank background ROIs (Figure 11C). Subtraction from tumor total radiant efficiency values automatically corrected for ROI size differences between each tumor and the flank background ROI. Corrected fluorescence results showed an improved correlation with bioluminescence data for the same mice. In addition, background correction also improved data variability, decreasing group standard errors by ~30%.

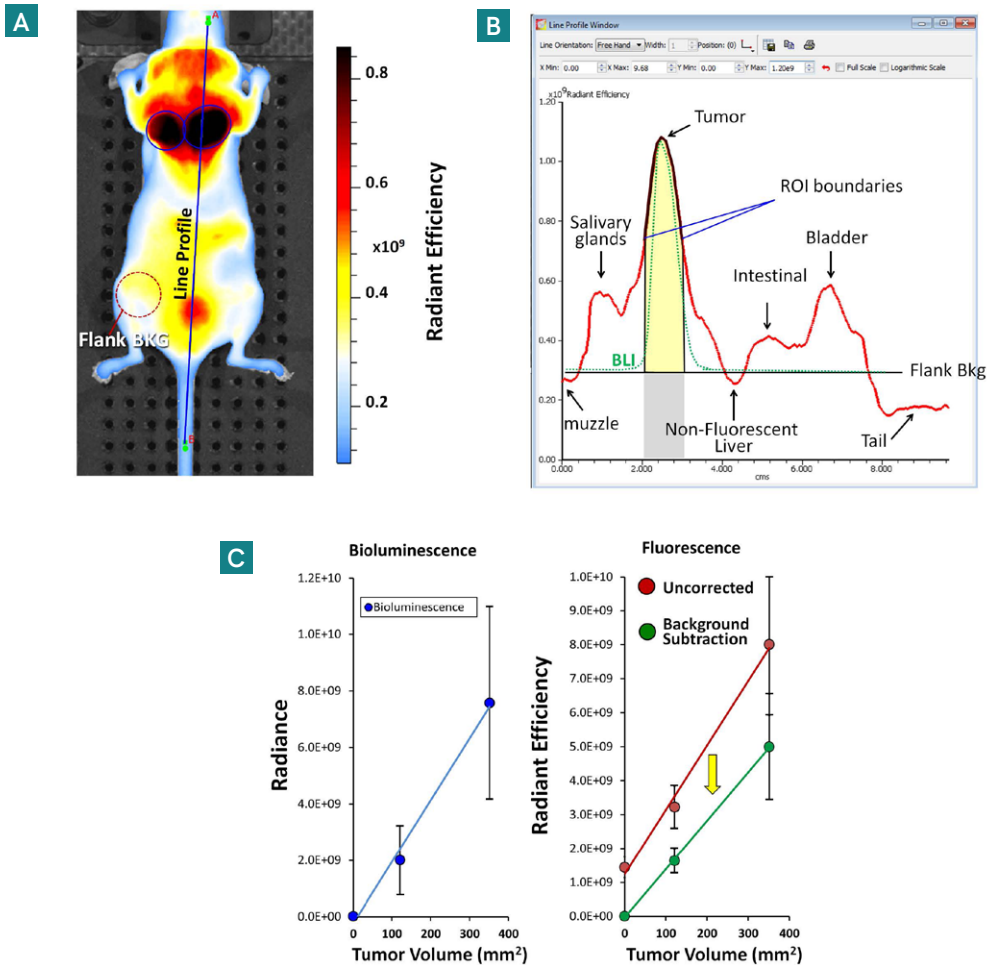


Figure 11: **Improved FLI profile with appropriate background subtraction in subcutaneous tumor model.** Some different background ROI approaches were used to further illustrate background variability in fluorescence imaging. (A) Mouse image representing background ROI placements and average signal. (B) Line profile with overlay of flank ROI average signal. (C) BLI and FLI (corrected and uncorrected) data to illustrate the improved pattern of quantitative fluorescence data with application of background subtraction.

Deep tissue 2D imaging and control animal background subtraction

Whereas analysis of subcutaneous tumor imaging models is optimal using ROIs that are closely tailored to size of the tumor, organ imaging requires the placement of ROIs that are identical and designed to capture the signal from the entire organ. In the example below, chemically-induced liver injury in mice (using thioacetamide to induce hepatocyte death) can be imaged using IVISense Annexin-V 750 (Figure 12AB). High doses of thioacetamide yield a roughly liver-sized fluorescent signal at the surface of the mouse, but lower treatment doses can yield signal of both lower intensity signal and smaller apparent size. In addition, for tissues like liver and kidney you expect some non-mechanistic control background since these organs are involved in general probe clearance/metabolism. This means that it is essential that your ultimate interpretation takes this into account.

Subtraction of control mouse background improves data interpretation. Using the internal flank background ROIs for *in vivo* datasets yielded approximately 0.18 x 10^9 (average radiant efficiency), control livers showing an average of about 0.22 x 10^9. This is illustrated by the superimposition of the background line on the line profiles of the four mice (Figure 12C). *Ex vivo* datasets used control liver signal for data correction.

Background subtraction (in both *in vivo* and *ex vivo* datasets) removed 35-55% of the total signal from the 300 and 100 mg/kg mice and 92-95% of the signal from the 0 and 30 mg/kg mice (Fig 12D). Background subtracted results place control and low dose mice at the origin of the graph (Figure 12E), aligning the graphical representation well with the interpretation.

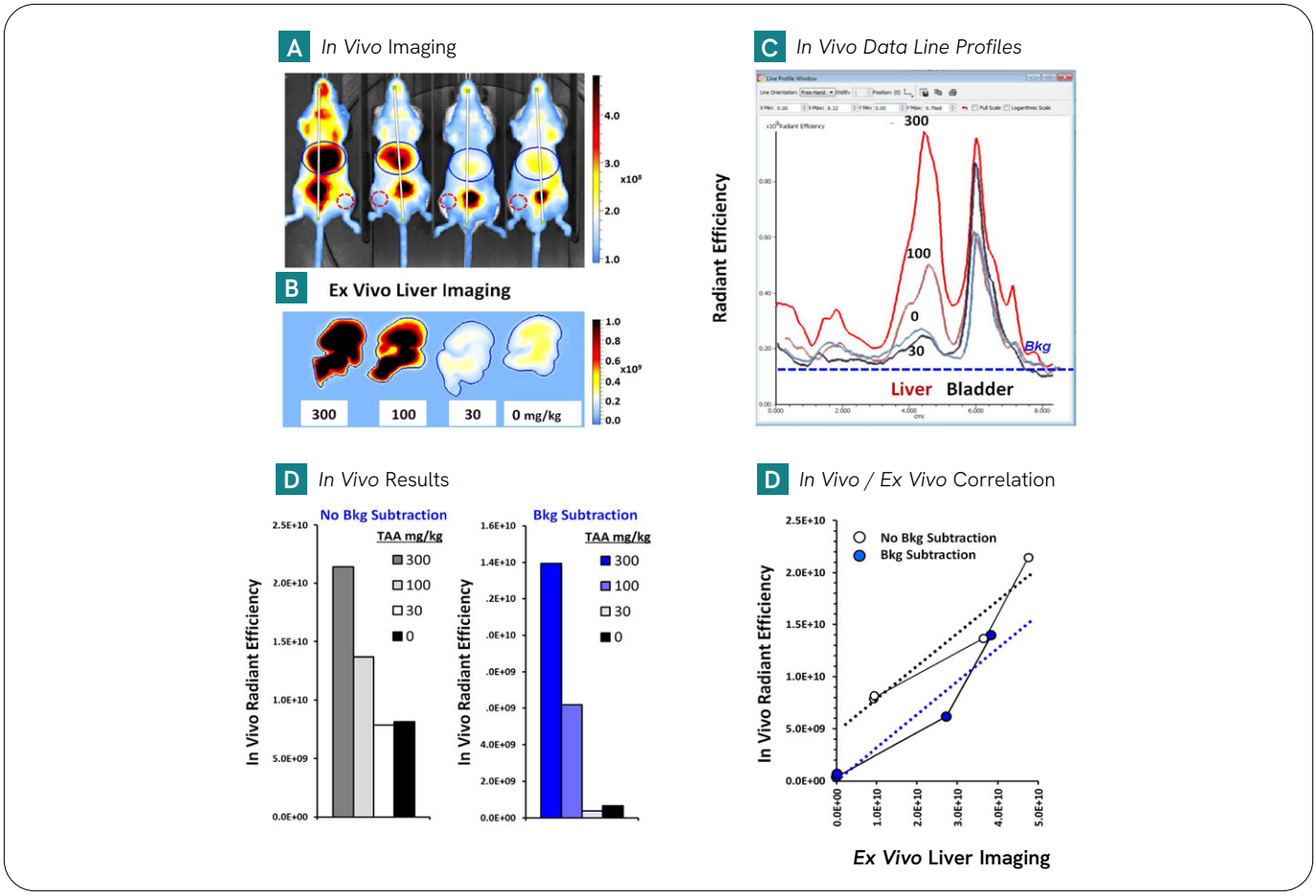


Figure 12: **Improved FLI profile with appropriate background subtraction in deep tissue model.** Representative thioacetamide (TAA) treated mice were imaged *in vivo* (A) and liver tissue *ex vivo* (B) with IVISense Annexin-V 750 to detect TAA-induced liver damage at different doses. C. A line profile was generated for each representative mouse to illustrate biodistribution profiles across liver and non-liver body regions. D & E. Without background correction, signal does not go to baseline, and the liver signal in control and 30 mg/kg treated mice appears to be 40% of that in 300 mg/kg treated mice. Background subtraction supports a different conclusion in which liver signal in control mice is 5-8% of that in 300 mg/kg treated mice.

Table 2: Suggested background ROI approaches by application.

Type of animal model	Preferred background ROI approach
Discrete FL Site	**Internal** – contralateral region or flank
Multiple FL Site	**Control Animal** – single or matching ROIs, manual subtraction of control group mean from each test ROI
Single Paw FL	**Internal** – contralateral ROI
Multiple Paw FL	**Control Animal** – matching ROI, manual subtraction of control group mean from each test ROI
Liver or Kidney FL	**Internal or Control Animal** – either internal flank or matching organ ROIs, automatic internal subtraction or manual subtraction of control group mean from each test ROI

Conclusions

Epifluorescence (2D) imaging offers tremendous tools and probes with which to study biological changes in living animals associated with disease progression or treatment. However, 2D fluorescence imaging requires strategies to accurately compensate for mechanistic or non-mechanistic fluorescence background; without proper fluorescence background correction, results may either underestimate biological changes or reduce sensitivity in the detection of lower intensity signal. This means that it is important to develop an objective background subtraction strategy that can be applied to fluorescence imaging datasets.

This technical note provides IVIS epifluorescence users with a quick overview of practical considerations for defining and applying background corrections to improve fluorescence quantification and interpretation. Additional supporting references are available to help to guide researchers in the basics of ROI drawing, setting up automatic background subtraction, and other imaging considerations.

References

Drawing ROIs. Revvity Technical Note. https://www.revvity.com/

Subtracting Background ROI from a Sequence. Revvity Technical Note. https://www.revvity.com/

Optical Imaging on the IVIS SpectrumCT System: General and Technical Considerations for 2D and 3D Imaging. Jen-Chieh Tseng, Kristine O. Vasquez, & Jeffrey D. Peterson. Revvity Technical Note. https://www.revvity.com/

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