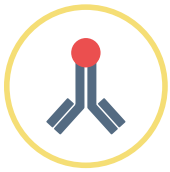
10 TIPS and TRICKS for IN VIVO imaging

spectral instruments imaging



CHOOSE REPORTERS THAT SUPPORT EXPERIMENTAL OBJECTIVES

Popular bioluminescent reporters expressed in cellular targets (e.g., luciferase enzymes), provide superior optical signal-to-noise ratio (SNR) data, and are typically the best option for studies using a single, cellular target. While fluorescent reporters have lower SNR, they collectively have distinct, Ex/Em spectra across the entire optical spectrum (from ~360 nm to 1000 nm). This allows FLI experiments to be multi-channel, and multi-targeted. This FLI approach is supported by commercially available kits that can label biological agents, including small molecules, antibodies, and cells. When setting up single or dual target FLI studies, near infrared (NIR, 690 nm to 1000 nm) fluorescent reporters are strongly recommended. NIR fluorescent probes have superior tissue penetration (>1 cm) and relatively good SNR, as their longer-wavelength, NIR light interacts minimally with tissues. Fluorescent reporters are also ideal for conducting sensitive ex vivo major organ biodistribution evaluations. Finally, some powerful experiment set-ups can be achieved by using both BLI and FLI in a single model. For instance, the relative locations of bioluminescent tumor cells and fluorescent therapeutic molecules can be assessed in a trimodal BLI, FLI and photo overlay image. Alternatively, with bioluminescence resonance energy transfer (BRET) imaging, luciferase enzyme and NIR probe co-expression enables the resonance energy of luciferase activity to excite the NIR fluorescent probe. This leads to NIR FLI with the low background, high SNR of BLI.
Yes, cool stuff is out there!

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4240620/pdf/nihms345603.pdf

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5682356/pdf/expanim-66-437.pdf

MINIMIZE ANIMAL HAIR AND SKIN INTERFERENCE

Dark animal hair and skin pigments absorb and scatter both incoming and outing light.
When possible, use hairless, albino or Hr mutant animal strains. If genetic background or immunocompetency status of the model does not allow this, then remove hair mechanically before imaging, by either shaving or using depilatory cream on anesthetized animals. Proceed carefully, and wash treated area when done. It is best to shave or depilate 24 hours prior to imaging, as the mild skin inflammation from hair removal can affect the biodistribution and/or activation of inflammation-targeted probes.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5682356/pdf/expanim-66-437.pdf

https://www.jidonline.org/article/S0022-202X(16)32464-2/pdf

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4240620/pdf/nihms345603.pdf

PLACE ANIMAL ON AN AUTOFLUORESCENCE-FREE DIET

In FLI, animal gut NIR autofluorescence will occur if the animal diet contains alfalfa, a plant material rich in chlorophyll. To avoid chlorophyll-based autofluorescence (at ~700 nm), switch animals to an alfalfa-free diet at least a week prior to imaging.

https://journals.sagepub.com/doi/full/10.2310/7290.2008.0003

CONSIDER ANIMAL ORIENTATION

Optical *in vivo* signal is attenuated by tissue. The deeper the source of light in an animal model, the greater the signal attenuation. To achieve maximum model sensitivity, identify the animal orientation giving highest signal intensity. One can take images from multiple positions to determine the best animal orientation. Dividers between animals are strongly advised as they will prevent confounding reflectance signals, i.e., secondary signals that occur when a strong signal from one animal reflects off the surface of an adjacent animal.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4240620/pdf/nihms345603.pdf

TRICKS FOR OPTIMIZING SNR IN BLI AND FLI

Generally, image signal-to-noise ratio (SNR) can be optimized by adjusting binning and/or exposure time. In BLI, take an initial image using moderate binning (4x4), and a short exposure time (5 seconds). If no signal is detected, then use higher binning (8x8 or 16x16), and longer exposure times (starting with 60 seconds, and working out to as long as 600 seconds, if needed).
If using D-luciferin for BLI in mice, be sure to prepare a fresh solution on the day of imaging, and give an IP dose of 150 mg/kg. This dose level is designed to achieve a period of luciferase saturation kinetics in most mouse models. For FLI, the best SNR is usually achieved by moderate to high binning (4x4 or 8x8), and exposure times that are short to moderate (5 to 30 seconds). Avoid long exposure times in FLI, as this can lead to elevated background noise (due mostly to tissue autofluoresecence).

IN BLI, CONSIDER DISEASE LOCATION WHEN SELECTING ROUTE OF SUBSTRATE INJECTION

Most frequently, D-luciferin is injected by an IP route, and this is ideal for systemic and subcutaneous disease models. However, for IP disease models, SC injections have been shown to give the best resulting images, as IP injections can lead to an artificially elevated IP bioluminescent signal. Additionally, with IP injections there is an elevated risk of inadvertently injecting substrate into an organ rather than into the peritoneal cavity. Given that the failure rate of SC injections is low, one could adopt this as the default route of substrate injection. The IV injection route yields brighter images, but it can be tricky to measure as the windows of peak signal are typically only 2-5 minutes post injection.

https://link.springer.com/article/10.1007/s00259-008-1022-8

https://www.goldbio.com/documents/1068/Luciferin%20in%20vivo%20handbook.pdf

CLEAN MICE AND MACHINE PRIOR TO IMAGING

Animal bedding, chow, and dander can create background phosphorescence signal. So prior to imaging, be sure to wipe animal paws and clean the imaging stage. This is best done using a tissue wipe moistened with70% ethanol.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4240620/pdf/nihms345603.pdf

ESTABLISH A BIOLUMINESCENT SIGNAL KINETICS CURVE

Bioluminescent signal intensity is a function of luciferase substrate kinetics. These kinetics are generally tissue dependent and can also vary over time, as a result of disease-related pathologies and physiologies. In BLI studies, one should identify and compare peak bioluminescent values over the time course of the study. This is best done by establishing bioluminescent kinetic curves at each imaging time point. Such curves are made by injecting, anesthetizing and then imaging animals over a span of several time points post-luciferin injection. Typically, imaging starts at 5 minutes post injection, is repeated every 5-10 minutes, and completed once signal intensities start to decline.

https://www.goldbio.com/documents/1068/Luciferin%20in%20vivo%20handbook.pdf

AVOID PIXEL SENSOR SATURATION WHEN IMAGING

Most optical imaging systems use cooled -90˚C CCD sensors, where each pixel in the sensor has the same, set maximum capacity to store signal information. Essentially, each pixel can be thought of like a "bucket," capable of holding a set amount of "water." If the bucket is over filled, then the water (or signal data) is lost and not recorded. If a given CCD sensor has 16-bit pixels, this maximum amount of "water" or data storage is 2^{16} bits of information, i.e., 65,535 gray scales of signal intensity. Thus, image intensities should stay within the range of 600-65,000 counts per pixel, to be both adequately above background, and below pixel saturation. If pixels saturate during imaging, the reported signal radiance values (photons/sec/cm^2/sr) will be erroneously low. Pixel saturation can easily be avoided by shortening exposure times, reducing binning, and/or by elevating f-stop.

USE THE POWER OF ABSOLUTE CALIBRATION

The optical signal intensity of a target can rise and fall over the time course of a study. Accordingly, camera settings may need to be adjusted in order to maintain good SNR or to avoid pixel saturation (see Tip #9). This is fine. It is OK to change the camera settings used to image a target. Why? Because all signal data is absolutely calibrated and then presented in units of radiance (photons/sec/cm^2/sr). The process of absolute calibration is driven by a set of system software algorithms that account for all sources of signal variability due to the imaging device.
These sources include not only camera settings used (exposure time, binning, FoV, and f-stop), but also various forms of CCD sensor noise and anomalies (dark current noise, camera bias, cosmic rays, CCD pixel imperfections, etc.) that may be present at the time of imaging. Bottom line: As a result of absolute calibration, any signal variability observed will be due to real changes in the target, and not due to any changes associated with the imaging technology.