HOW TO **QUANTIFY**
BIOLUMINESCENT CELLS
IN YOUR **ANIMAL MODELS**

INTRODUCTION:

In vivo optical imaging is a semi-quantitative imaging modality, where signal intensities correlate positively with the number of reporter molecules. Consequently, optical data can be used to answer experimental questions such as: *"What are the relative efficacies of two drug treatments against a given tumor cell line?"* The drug treatment leading to the greatest reduction in optical signal intensity will be considered the most effective therapeutic. Beyond such efficacy studies, there are instances in which investigators will want to know the exact correlation between signal intensity and cell number for a given reporter construct, either *in vitro* or *in vivo*. This data can be acquired, but it does require a bit of extra work. Essentially, one needs to create a standard curve of optical signal intensity vs. cell number. Below is a general protocol of how to generate both in vitro and in vivo standard curves (optical signal intensity vs. cell number) for bioluminescent reporter systems.

*NOTE: Theoretically, the procedure below can be applied to either bioluminescent or fluorescent reporter models. In bioluminescent models, you will be defining a correlation between optical signal vs. number of **living** target cells present, given that the functionality of bioluminescent enzymes (typically luciferases) will require not only their specific substrate, but also oxygen and cellular ATP, the latter being present in only living cells. In fluorescent studies, you are typically defining a correlation between optical signal and the total number of fluorescently tagged cells. Such labeled cells may be **alive, dead or dying** as fluorophore functionality (the absorbance and emission of light) does not require cellular ATP. To exemplify how in vivo standard curves can be made, we have opted here to use a general bioluminescent oncology model.*

GENERAL PROTOCOL:

FIRST, MAKE AN *IN VITRO* STANDARD CURVE

These preliminary experiments will allow you to confirm up-front that the transduced tumor cell line of interest generates a good bioluminescent signal intensity. Furthermore, the *in vitro* standard curve will allow you to approximate the target cell number needed for producing *in vivo* standard curves (see below). In general terms, *in vitro* standard curve data are acquired by titrating out a cell preparation into cell medium, in triplicate, at least (n ≥ 3), and then measuring optical signal intensity of each titration in units of total photons/sec. You can then plot mean intensity data (mean of total photons/sec ± standard deviation) vs. cell numbers to produce an *in vitro* standard curve.

DETAILS:

- Harvest your luciferase-transduced tumor cells of interest from tissue culture, tumor biopsy, or tumor cell-burdened organ (*e.g.* T-cells from spleen), and determine cells/mL values through the use of a hemocytometer and standard light microscope.

- Perform all cell dilution series (titrations) in black, non-fluorescing, non-reflective plastic flat-bottom 96-well plates. Additionally, it is best to use 96-well plates that have a low-binding surface to minimize cell loss during serial dilutions.

- You should ideally run two *in vitro* titrations. In the first run, you can use 1:10 dilutions. This will give you an approximate correlation between signal intensity and cell number over a multiple-log range of cells/well. In the second titration series, you can use shallow, 1:1 dilutions. This will allow you to determine the exact threshold of bioluminescent cell detection.

- For the first 1:10 titration series, you can prepare and work with a starting cell solution that will give 10e6 cells/well. You can then titrate across the 96-well plate, down to a concentration of ≤ 10e1 cells/well. When imaging this set of titration samples with a Lago X, Ami HTX or Kino you can use short exposure times and modest binning (e.g., 5-10 sec, with 2x2 or 4x4 binning). Again, the data of this titration series will define an approximate correlation of bioluminescent signal intensity vs. cell number, and you will need a second, 1:1 titration series to accurately define a cell detection threshold.

- With your second 96-well titration study, you can start at 1 to 2 cell dilutions above the minimum detection concentration (determined by the first titration series), and then run a 1:1 serial dilution across your 96-well plate. For this second set of titration samples with fewer cells/well, you can use longer exposure times and higher binning (e.g., 60-300 sec, 8x8 binning). Again, the data from this second *in vitro* titration will allow you to determine an accurate detection threshold of cells/well.

*NOTE: The range of cells/well values to use in your **in vivo** titrations should be guided by results from your preliminary, **in vitro** standard curves (above). It is a given that **in vivo** optical signal is attenuated by tissues, specifically by oxygenated hemoglobin and melanin pigment. So, when performing **in vivo** cell titration studies, you will typically use a higher range of cells/well values than that used in the **in vitro** titrations (see below).*

SECOND, MAKE AN *IN VIVO*, ORTHOTOPIC STANDARD CURVE:

In general, you can serially dilute cells, and then immediately inject your cell samples into an animal model organ or body compartment of interest. The optical signals from challenged, test animals will then be imaged by a Lago X, Ami HTX or Kino system.

DETAILS:

- Harvest luciferase-transduced tumor cells of interest from tissue culture, tumor biopsy, or tumor cell-burdened organ (*e.g.* T-cells from spleen), and determine cells/mL values through the use of a hemocytometer and standard light microscope.

- Given prior *in vitro* standard curve data (see above), prepare a stock cell solution to be used in the *in vivo* titration. Typically, your stock cell solution for *in vivo* titration can be 10- to 100-fold that of the starting concentration used in the *in vitro* 1:1 dilution series (see above).

- Serially titrate cells into a physiologically acceptable solvent (e.g. PBS), over an appropriate concentration range, perhaps down the detection level observed *in vitro*.

- Prepare enough of each cell concentration so that you can perform several orthotopic bolus injections per concentration. Typically, n = 5 per cell concentration is recommended, as it will lead to statistically tight data.

- Inject cell titration samples into your major organ/tissue site of interest. Regardless of the site of injection, always aim to minimize the volume and speed of your bolus injection. This will minimize any site disturbance and/or any cell leakage from site. You may choose to suspend cells in a Matrigel preparation to have a better retention of cells at the injection site.

- Image animals immediately after they receive the bolus cell injection, using a [Lago X](#), [Ami HTX](#) or [Kino](#) imaging system.

- Run a Region of Interest (ROI) analysis of all optical signal data (using units of total photons/sec).

- For each dilution of the titration series, determine and plot mean signal intensity (mean total photons/sec +/- standard deviation) *vs.* known cell number data.

- With the resulting *in vivo*, orthotopic standard curve, you will now be able to translate observed bioluminescent signal values into *in vivo*, viable target cell loads. Remember, different bioluminescent reporters, cell lines, orthotopic sites, and mouse strains will alter these *in vivo* standard curves. Any time such parameters are altered in a model, a new *in vivo* standard curve should be determined.

Important Note:

When doing ROI analyses, we recommend that bioluminescent signals be quantified in units of "total emission" (total photons/sec) and not in units of "mean radiance" (mean photons/sec/cm^2/sr). The reason for this is simple: Mean radiance is inversely correlated with the amount of background area (i.e., low signal intensity area) included in drawn ROIs. If there is variability in the ratio of signal area to background area in drawn ROIs, then there will be variability in mean radiance values that is not biologically based, but is instead due to the way the ROIs were drawn. Importantly, and in contrast, total emission values remain consistent and unaffected by any such variability in how ROIs are drawn.