Kinetic Analysis of Bioluminescent Sources

Acquiring the most accurate quantitation of your bioluminescent sources requires a close understanding of the underlying kinetics involved in producing and capturing the detected light. After injection, the substrate for your bioluminescent probe will diffuse through the body of your subject eventually coming in contact with the luciferase enzyme in your target cells. In the case of Firefly luciferase, D-luciferin is catalyzed to oxyluciferin in the presence of ATP, Mg^{2+}, and oxygen producing light as a consequence. It is this light that is measured and used to accurately quantify your samples *in vivo*. The diffusion of D-luciferin is dependent on several factors including but not limited to method of injection, metabolism, and tissue localization of your source.

Commonly utilized injection methods include intraperitoneal, subcutaneous and intravenous with the first two seeing the most widespread usage. Intraperitoneal is by far the most commonly used method in which a volume of your substrate is injected into the peritoneal cavity. With this method, the threat of injecting the substrate into an organ instead of the peritoneal cavity is very real especially in severely diseased and emaciated subjects. This often results in confusing data where a source simply fails to luminescence or is extremely dim compared to other mice/days. In this case, the most accurate method for rectify this botched injection is to wait until complete clearance of the injected luciferin approximately 2-3 hours and then give a second injection. For this reason, many are switching to subcutaneous injections into the scruff of the neck as the failure rate is much lower once this procedure is mastered. See ***Inoue et. al., 2009, Eur. J. Nucl. Med. Mol. Imaging 36:771–779*** for more information on comparing subcutaneous with intraperitoneal injection routes. Intravenous injections although brighter typically peak within a minute or two and the imaging window will be very small. Therefore this method is not encouraged as the window for error is much greater.

Metabolism issues are often overlooked when setting up new models but can affect diffusion of your substrate dramatically. Some of the most commonly observed issues occur due to changes in animal strain and animal handling procedures. Simply, multiple animal strains may have different kinetic curves due to differing metabolic rates. Furthermore, how you handle the animals is of major consequence. Do you inject the substrate before or after anesthesia? If you anesthetize after injection, is the time kept absolutely consistent? Are the animals kept warm using a heating pad or by being placed directly on the IVIS stage until the time of image capture? In essence as long as the answers to these questions remain the same for EACH image capture, any answer will suffice. As for recommendations, we prefer to inject before the animals are anesthetized and we certainly recommend keeping the animals warm after anesthesia. Anesthesia can slow the metabolism to a point that the animals become hypothermic which can place a major stress on your animals, thereby potentially altering experimental outcome by compromising the health of the animals.

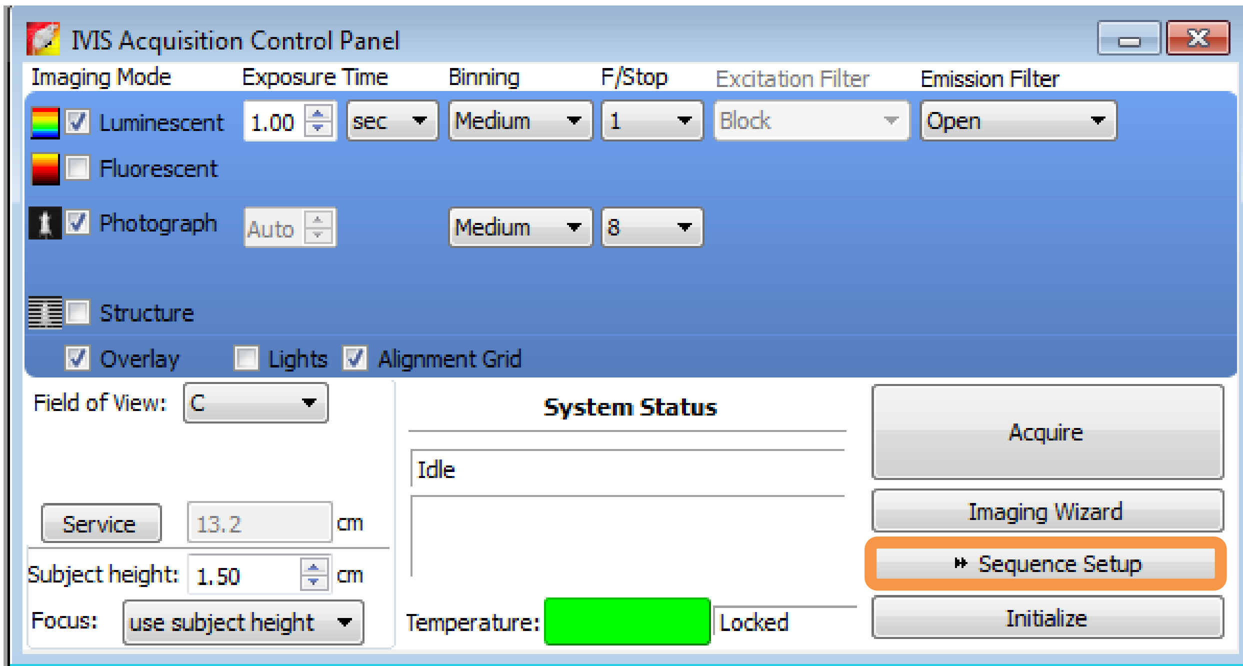
D-luciferin is known to traverse the cell membrane and crosses the blood brain barrier but the time that is required to do this will vary depending on proximity to injection site. Certainly the further the substrate must diffuse the longer it will take to see peak intensity. ***Burgos et. al. 2003 BioTechniques 34:1184-1188*** perform a nice comparison of orthotopic flank tumors and brain tumors. Lastly, once the substrate reaches the source the amount of enzyme i.e. copy number will affect the lower limit of detectability and can cause a shift in the curve. Always perform a new kinetic curve for each new cell line that is created in your lab.

A basic protocol to attain your kinetic curve follows:

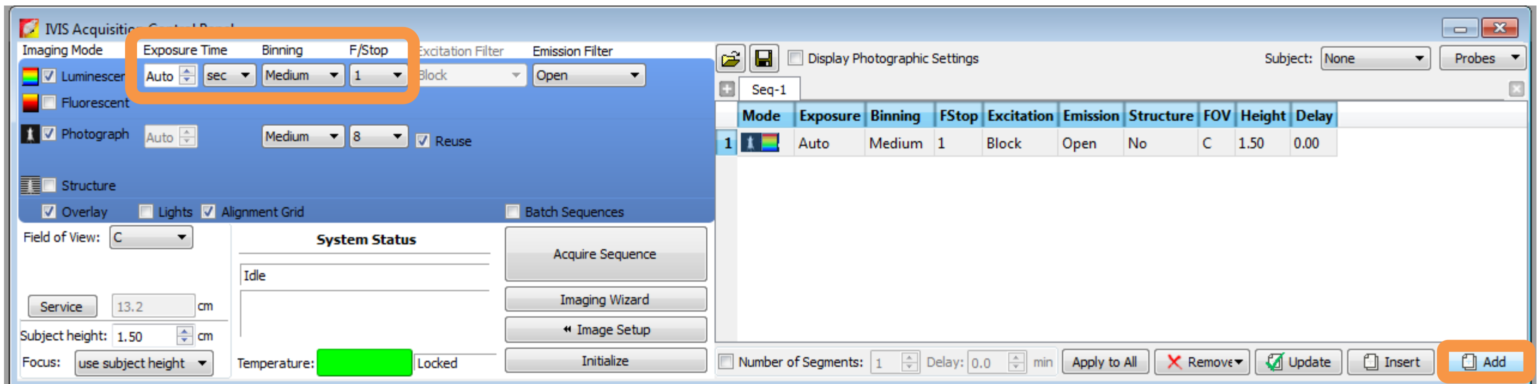
Required items – D-luciferin solution sterile filtered in DPBS, 25 gauge needle, 1cc syringe, and timer

Note – Kinetic curves need not be performed with large groups of animals. Typically 3-5 will suffice.

1. Before you begin the procedure it is a good idea to set up the software and prepare it to capture your kinetic sequence. Start and initialize Living Image.
2. Select **Sequence Setup** on the IVIS Acquisition Control Panel.



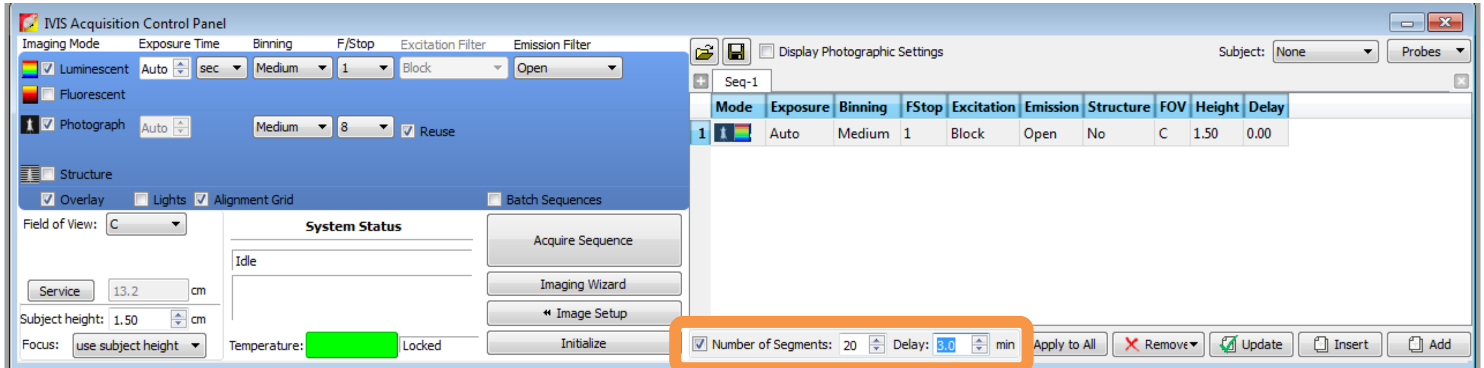
3. Select the appropriate sensitivity settings. **Autoexposure** will work well for exposure time as it will prevent you from saturating your sources in later time points and default settings of **Medium binning** and **F/Stop 1** are recommended as a starting point. Autoexposure will adjust these as needed during the acquisition to attain the desired count values.



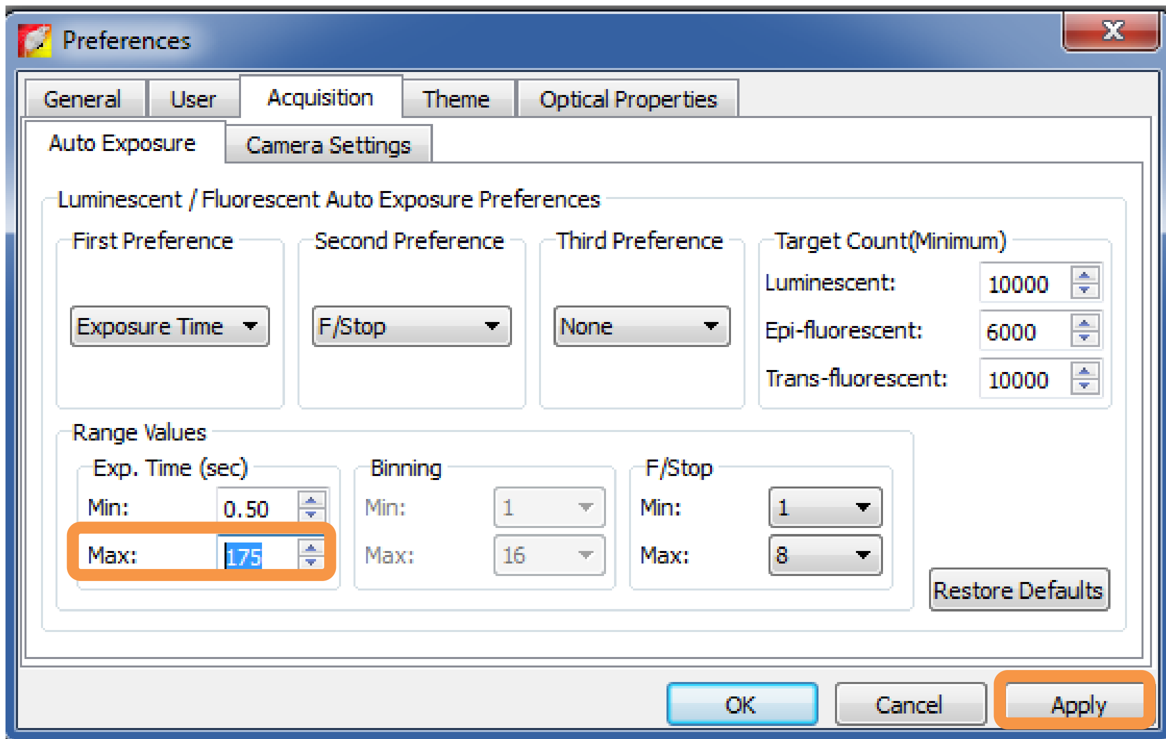
4. When you are satisfied with your settings select **Add** on the bottom right of the Sequence Editor window and that image will be added to the sequence.
5. We need to make sure that the image acquisition will now repeat itself for a defined number of times with a delay between the acquisitions. It is recommended that you map your curve for approximately 1 hour taking images every 2-5 minutes. For dimmer signals, we can extend the exposure time and for brighter

models we can decrease the exposure time. For example if we wanted to acquire one image every three minutes for an hour, we would select the **Number of Segments** check box on the bottom left hand corner of the Sequence Editor window, add a delay of 3 minutes with a number of segments equal to 20. That means we will acquire 20 images with a delay of 3 minutes between each image equaling a total acquisition time of 60 minutes. Image 1 starts the moment you select Acquire Sequence.

6. It is important to understand that autoexposure will override your image capture delay. We want to

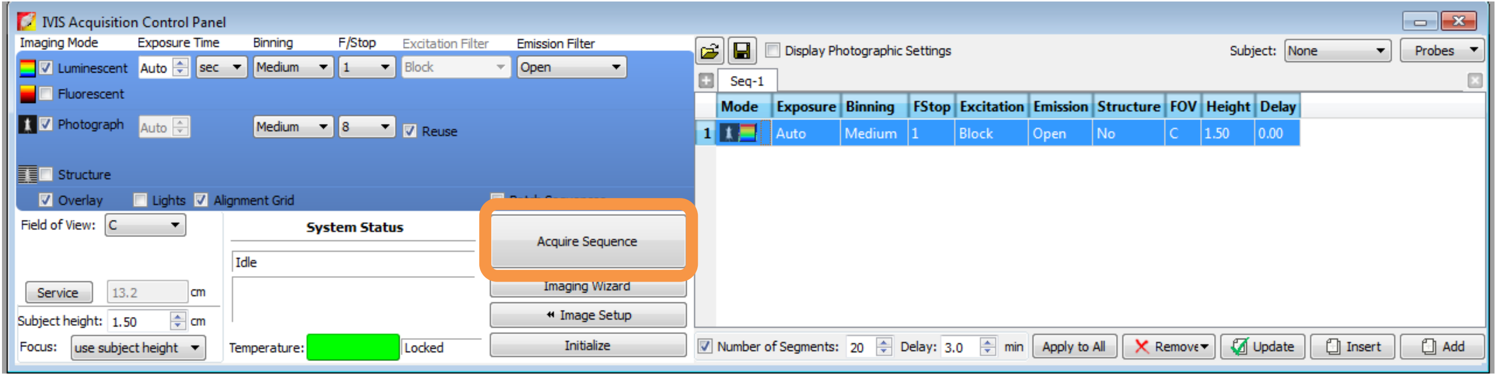


allow the software the flexibility to obtain maximum sensitivity with the autoexposure feature while preventing it from overriding our set time interval. Default autoexposure settings max at 1 minute so we do not risk overriding in this particular case. However in the first few captures, we may need more than 1 minute exposure time to obtain the desired count values. I would suggest changing max exposure time to the delay time minus 5 seconds. The five seconds allows for image read time and motor movements. To change this go to **Edit>Preferences>Acquisition>Autoexposure**. Change the max exposure time (shown in seconds) accordingly. In this example, our max time would be 175 seconds.

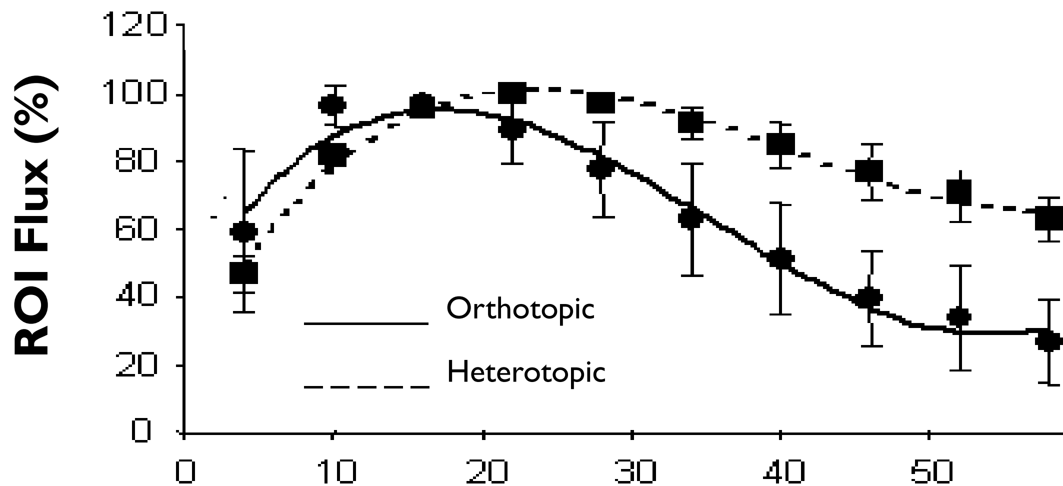


7. Click **Apply** when you are done.
8. The software is now ready for acquisition and you can begin injection of your animals.

9. Inject D-luciferin at a concentration of 150 mg/kg body weight. An example with a 10 gram mouse would be to prepare stock solutions at 15 mg/ml and inject 100 µl.
10. Inject the substrate at the desired location – I.P. or S.C.
11. Wait three minutes, and then sedate the animal in the supplied chamber.
12. **As soon** as the animals are sedated, transfer them to the IVIS chamber and the 37°C stage using the supplied manifold and nose cones to keep the animals sedated and warm.
13. 5 minutes after injection start the first capture by clicking **Acquire Sequence**.



14. The software will acquire images for approximately one hour. With 2% isofluorane, healthy animals can be sedated for up to an hour with no issues.
15. Data should be plotted at Total Flux versus Time or Percentage of Max signal versus Time.



A good example as seen above can be found in the ***Burgos et. al. 2003 BioTechniques 34:1184-118.*** Typical kinetic curves will have a sharp increase followed by a plateau of expression and a gradual reduction of signal out to and beyond an hour. Plateaus tend to be 10-20 minutes in length depending on the method of injection. For optimal quantitative results, ALWAYS determine and image in the plateau of your kinetic curve. Any other points on the curve are prone to error and can cause false interpretations and large standard error.

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