TransMedRI

Workshop on

SMALL ANIMAL IMAGING

March 15th & 16th 2012

Faculty of Medicine, University of Rijeka

Rijeka, Croatia

TransMedRI

CAPACITIES

Workshop in Small Animal Imaging

Data Analysis Day 1

Background

As we have learned during the lectures, imaging of small laboratory animals can be performed using a variety of techniques. Limitations are set by the clinical parameters that the researcher wants to investigate and the physical methods that the researcher is applying. For example; in two-photon technologies, the researcher wants to image the movement of cells (clinical parameter) using fluorescent probes (experimental method). This will require a completely different experimental setup compared to when one wants to image bone density using X-Ray.

In this session, we will use our Carestream MS FX Pro© *in vivo* imager. This machine is capable of imaging Fluorescence, (Bio/Chemi-) Luminescence, X-rays and Radio-isotopes. In this demonstration we will show imaging of tumors and virus expressing luciferase and tumors expressing fluorescent markers. The primary parameter will be therefore photon emission by the experimental subjects (mice). We will combine this technique with X-ray to be able to co-localize the tumor/virus within the animal.
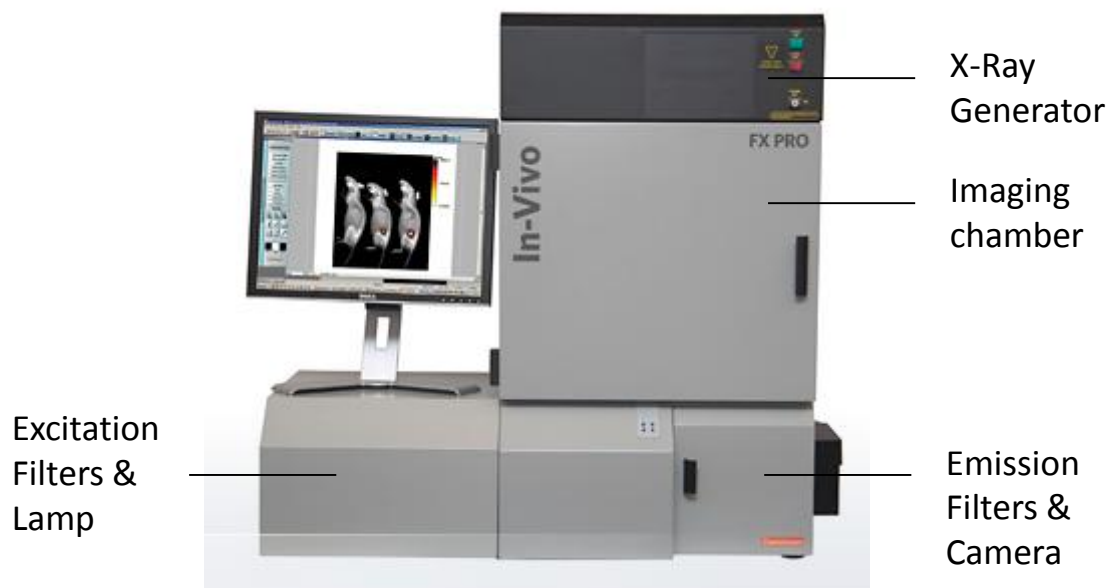
During the practical part of this workshop we will split the group into two subgroups. One group will watch the demonstration of how we anesthetize the animals, prepare them and image the animals. The other group will get the images of a previous measurement and will learn how to analyze data and how to generate a picture of imaged animals for publication.

Data Analysis

Today we will analyze images of three separate experiments:

1. Mice injected with Luciferase-expressing cytomegalovirus
2. Mice injected with Luciferase-expressing B16 melanoma
3. Mice injected with Luciferase-expressing U87 astrocytoma

We will first go through the analysis of experiment 1 step by step. Next, you will be required to generate data of experiments 2 and 3, by using identical methods.

Experiment 1:

Background

Wild type C57BL/6 mice were depleted from NK cells by i.p. injection of αNK1.1 antibodies. 24 h after depletion, mice were infected with 5 x 10^5 PFU mCMV-Luc. Four days after infection, mice were anesthetized with isofluoran gas, hair of the abdomen was removed with depilation cream (we recommend Veet© for the sensitive skin) and mice were injected with 3 mg Luciferin. Mice were rested for 10 min to allow the distribution of luciferin through the body. Next, light emission was imaged for 10 minutes. Subsequently, a reflection image (also named white light image; 0.2 s) as well as an X-ray image (1 min) were performed. Finally, an X-ray correction is applied by the automated epi-field flattening methode.

Analysis Procedure:

1. Open Carestream MI SE
2. First, we will generate a representative picture. Next, we will quantify the luciferase expression and put it in a graph. Open the folder containing the files of non-depleted mice, using:

    File/open/Desktop/Workshop/Experiment1/mCMV Non-depleted

3. In this file, you see two files; one corresponding with the X-Ray and one with the luminescence measurement. Open the file 'X-Ray mCMV Non-depleted'.
4. The file will open an X-ray taken of the mice, plus a little window on the right ('Image display'), with which you will be able to optimize your picture.
5. On the left hand side of the screen, select 'Image'. Use the magnifying glasses to zoom in or out.
6. Go to the Image Display and select 'invert' (or de-select it) to acquire an image in which the animal is dark and the background white.
7. Click the little blue cross to open the advanced settings.
8. Adjust the image settings to your liking. In this image it is sufficient to change only the 'Min' and 'Max'. Use, for example a min of -400, max of 4000 and gamma of 0.9.
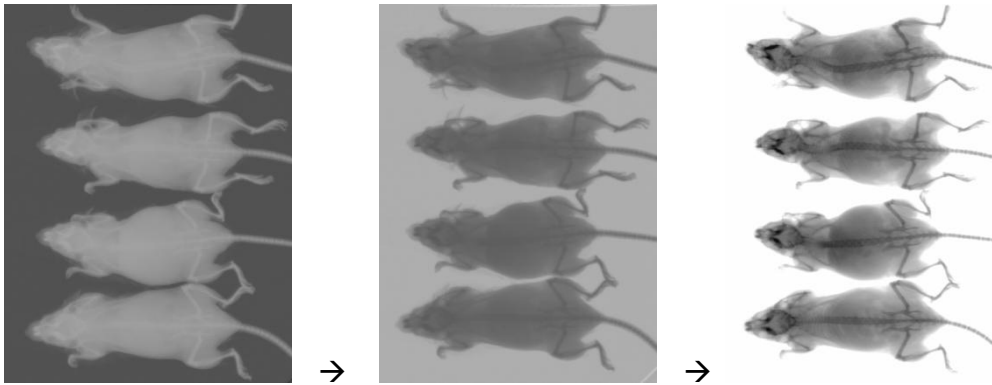
9. Save file, using

   File/save as/desktop/saved files/experiment 1

   Give the file a name, press save and OK

10. Next open the luminescence file named: 'Luminescence mCMV Non-depleted'
11. Now you see the image of the luminescence measurements.
12. If the Image Display window is not opened, open it using the button at the top of the screen.
13. Invert the display in such a way that the background is bright and the signal is dark.
14. Open the advanced settings to optimize the image.
15. First, adjust the 'Minimum' threshold until the background is white. This should be at a value of approximately 200 AU (Arbitrary Units).
16. Next, under the button 'Display' select 'Fire'.
17. Adjust the maximum in such a way that differences are optimally visualized. This should be at a value of approximately 1000, with Gamma at 1.
18. Finally, adjust the smoothness. Next to the button saying 'Median' there's a button saying '3x3'. Change this to 7x7 in order to smoothen the image.
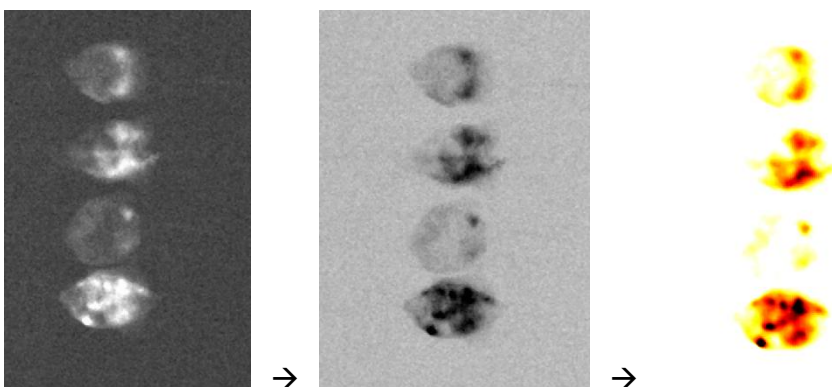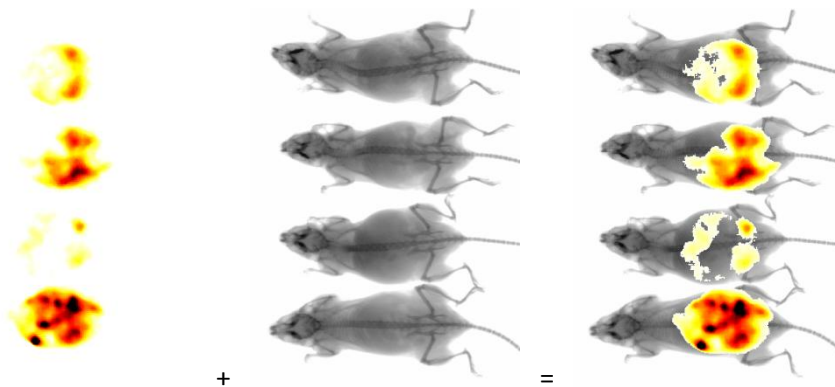

19. Save the file in the same folder as your X-Ray.
20. Next, open the X-ray image you just saved.
21. In the advanced Image Display, select 'Overlay' and subsequently 'Transparency'.
22. If this fails, make sure that you're putting the Luminescence file on the X-Ray file and not vise versa.

+   =

Next we show two separate possibilities how to export the generated images.

(i) The program allow exporting of the multilayered image by use of the function "Export" → "Image" → EPS file. Two files will be generated. One file with the background and one file with the overlayed image.

(ii) The second method to export this image, we be:

23. Zoom in on your image as much as possible, while still seeing all animals on the screen.
24. On the keyboard press 'ctrl' together with 'Prtscn'
25. Open Microsoft Paint under 'start menu' in windows.
26. On the keyboard press 'ctrl' together with 'v'
27. Crop the image to your preferred size.
28. Save the file in the Experiment 1 folder.
   (Note: You can procee the same way by using PowerPoint)

Next we will analyze the luminescence data. Go again to the MI program.

29. Choose the Luminescence Image file (Note: Do not use the X-ray image file!).
30. At the top of the screen, click the 'Navigation' button, to open the Navigation menu.
31. Next click 'Manual ROIs'

ROI stands for **R**egion **O**f **I**nterest. We are going to determine which are our ROIs in the image and subsequently quantify intensity of the signal. In this experiment we are interested in the total amount of virus per mouse. Therefore, our ROI should include all signal per mouse and the final data graph should represent all cumulative signal, corrected for background signal. The program is rather particular in its commands, so follow the protocol carefully.

32. Within the ROI menu, click on the red square ('ROI Rectangle')
33. Draw a square around the signal of one of your mice. Make sure that the (majority of the) outsides of the square are NOT within your signal. The rims of your square are going to determine your background. Everything in the square is ROI.
34. Next, put the cursor in the center of the ROI you just created, so that a hand appears. Click 4 times in the center to generate your ROI 5 times.
35. Go to the ROI menu and click on the little arrow to select the 'Pointer selection'.
36. Now, go back to the image and move the four ROIs so they fit around your signals.
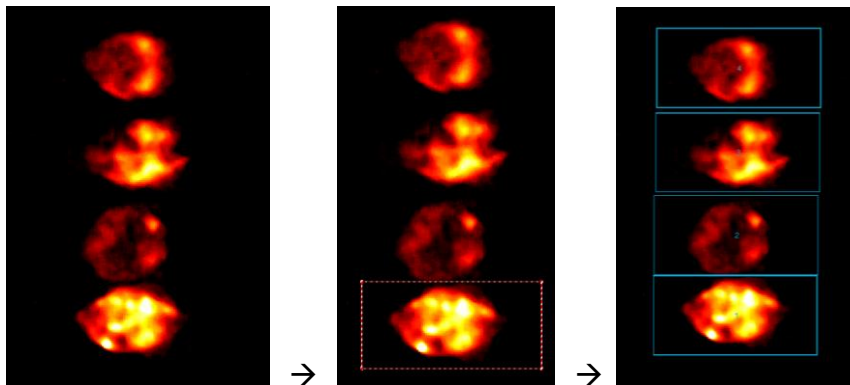
Next we will set the background. For the visual image, currently there appears to be no background. In reality, this is not the case; your background just has a value below the minimum threshold that we set. As you remember, we adjusted the threshold for minimal signal in such a way that the background on your screen was black (or white). For the data-analysis that background WILL be taken into consideration. The best background levels are the ones which are closest to your ROI. Therefore, the background will be determined *per ROI* by the median signal measured on border of the ROI (i.e. the blue lines). In other words, the computer will generate a median background signal *per pixel* based on the average signal it sees under the blue lines. Median is used instead of mean, in order to compensate for any specific signal that coincidentally overlaps with your ROI. After calculation of the median background signal, the program will subtract that signal *per pixel* of all the pixels within your ROI. This will generate the Netto intensity that we will use as our primary read-out. If you generated ROIs of different sizes, you have to compensate for this parameter. Since we generated ROIs of equal sizes, we don't have to do this.

37. In the ROI menu, click 'Set Background'.
38. Select 'Use the Median of each ROI parameter'.
39. Click OK.

Next, we will generate a template, so we can apply these ROIs to all our separate measurements.

40. In the ROI menu, click 'Templates'.
41. Click 'Save to Template'.
42. Name your file and save it in the 'Saved Files/Experiment 1' folder.

Now, we will acquire the data we generated.

43. On the top of the screen, select 'Analysis'
44. In this screen, click 'Select all'
45. On your keyboard, press 'ctrl' and 'c'.
46. Open Microsoft Excell.
47. Press 'ctrl' and 'v'.

In order to apply your ROI settings to the second measurement, use the following protocol:

48. Open the second luminescence measurement
49. Open the ROI menu and select 'Templates'

50. From the drop-down menu, select the template that you just generated and press 'Apply to current document'.
51. With the 'Pointer Selection' mode, move the squares so they fit over your samples.
52. Open 'Analysis' again and copy the data again to excel.
53. Use the Net Intensity as your primary parameter for the generation of graphs.

Exercises:

Experiment 1

1. Generate overlay pictures of the two groups of mice using the protocol described above.
2. Generate graphs in excel.
3. Calculate, using student t test, whether there is a significant difference between the two groups.

Experiment 2

Background

Mice were injected intravenously and subcutaneously with luciferase expressing B16 melanoma cells. When injected s.c. these cells generate a tumor that is restricted to the site of injection. When injected i.v. these cells infiltrate the lungs and generate tens to hundreds of 'metastase-like' tumors. Mice were injected with B16 cells three weeks previously, injected with luciferin and imaged. Data files can be found under File/open/Desktop/Workshop/Experiment2

Exercises:

1. Generate overlay pictures of i.v. and s.c. injected animals.
2. Generate graphs in excel.
3. Give the relative tumor size after i.v. and s.c. injection. Set the average of i.v. injected tumors as 1.
4. Calculate, using student t test, whether there is a significant difference between the two groups.

Experiment 3

Background

Mice were injected s.c. with luciferase expressing U87 astrocytoma cells. These cells are originally derived from a human brain cancer. Due to oncogenic transformation, they can form tumors anywhere in the body of immune-compromised mice. Five weeks ago, SCID mice (which have no adaptive immune system) were injected subcutaneously with U87 cells. Mice were injected with luciferin and imaged with identical settings as the B16 cells from the previous experiment. Measurements can be found under File/open/Desktop/Workshop/Experiment 3

Exercises

1. Generate overlay pictures of s.c. injected animals.
2. Generate a graph in excel.

Tumor growth may differ dramatically, based on the oncogenic transformations they have acquired and the tissues from which they were originally derived. Some cancers stimulate angiogenesis and generate well vascularized tumors, which greatly enhances their viability. Others grow completely without any structure or vascularization, which results in large necrotic areas within the tumor. Using the data of experiments 2 and 3 we will estimate the relative number of viable cells in the tumor. To calculate this, we need to provide three parameters: tumor size, luciferin expression per cell and average cell size. For simplicity you may assume that average cell size is identical between the two lines:

Luciferin expression was determined by flow cytometry:
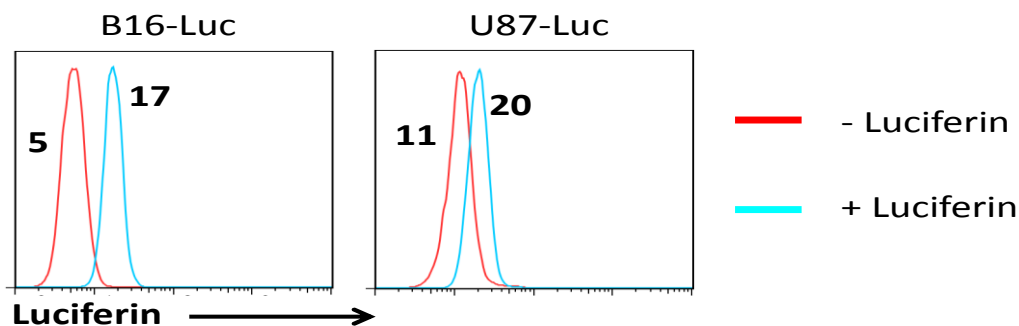
Tumor weight (gram):

	B16 - Luc	U87 - Luc
Tumor 1	1,2	0,5
Tumor 2	0,9	0,6
Tumor 3	1,1	0,6

3. Calculate relative luciferin expression as fold increase over background. Use Expression in U87 cells as 1.
4. Calculate relative number of viable (in this case luciferin expressing) cells per tumor. Take the value of U87 cells as 1.

Day 2

Spectral Unmixing Protocol for fluorescent *in vivo* images

1. Open the Carestream Multispectral Viewer Software.

2. Open the experiment (.xml) file under File/open/Desktop/Workshop/Experiment4

3. Select the X-ray image bar to view the whole mouse. Now you should have the mouse clearly visible with an overlay of the fluorescent signal.

4. Move the white cursor to find the wavelengths that have signal at different locations on the mouse. Check if locations without injected probe have significant background signal at any given wavelength. Also check the locations where probes with different wavelengths emit. You need to know which probe is applied at which location. Note; some probes may emit at two different wavelengths. Go through the wavelengths of the Wavelength selector curve. Are there background (not related to the probe) signals?

5. Now, we will create probe models. Go to "Unmixed images" on the Layers panel to the right, click "+" and enter a new model type name. Enter "691" in the Model Type field and "Far Red" in the box for Model Application.

6. Repeat for "761"; "Fur" and "Gut" (these latter two emit background signals that need to be subtracted from the specific signal).

7. Now we will model each probe in turn.

8. Return to the Layers panel and deselect all except 691 nm.

9. Mark the site with highest 691 nm specific signal on the mouse using the white cursor. This can be done by selecting the 691 nm wavelength (and unchecking all others) on the Layers panel and looking at the image to see where the white cursor needs to be moved.

10. On the 691 nm bar on the Layers panel; click the wheel "✳" to Edit the spectral model. Click on measure on the Wavelength selector field.

11. Put the red cursor on the specific signal (should align with white cursor).

12. Blue cursors need to go on the fur and on skin (mouse was shaved on the back to reveal some of the bare skin). Of note, for 691 nm, 791 nm and gut; we will select fur and skin as background, whereas for the fur probe model, the background will be the plate. **During the measurement mode, try to move the blue cursors so that the yellow line (specific signal) is as close to the bottom as possible on the non-relevant wavelengths (applies most of all to fur background; 540 nm).**

13. Now click "Model". For each application; one needs to model the field created by the measured values at the different wavelengths using a composite of several curves. The program first proposes one Gaussian curve with peak at the highest signal wavelength for each model. However, for some wavelengths; several peaks appear and need to be modeled. For this, double clicking on the green curve should give additional (accessory curves in white labeled P, 1 and 2, 3, n…). These may be moved left and right and changed in width to model the green field. Switching between the 1-, 2- and P- curves may be done by clicking the respective buttons for each. The sum of the three accessory curves will be the green curve, which will model the green field as closely as possible.

14. When an ideal curve fit is approached, click "optimize curve".

15. If the optimized curve presents with a less precise fit, click "restore".

16. Exit the dialog box to close the probe editor.

17. This process needs to be repeated for each probe model.

18. When all probes are accurately modeled, click "Unmix". The software now unmixes the signal for each probe.

19. Now, each Unmixed probe signal is exported to the Carestream MI Software by clicking "MI".

20. Zoom out image, using "Navigation", then "Image". Check "minifying glass".

21. Click again Navigation panel and choose Manual ROIs.

22. Next click 'Manual ROIs'

23. Within the ROI menu, click on the red square ('ROI Rectangle')

24. Draw a square around the signal of one of your mice. Make sure that the (majority of the) outsides of the square are NOT within your signal. The rims of your square are going to determine your background. Everything in the square is ROI.

25. Next, put the cursor in the center of the ROI you just created, so that a hand appears. Click in the center to generate your ROI.

26. In the ROI menu, click 'Set Background'.

27. Select 'Use the Median of each ROI parameter'.

28. Click OK.

Next, we will generate a template, so we can apply these ROIs to all our separate measurements.

29. In the ROI menu, click 'Templates'.

30. Click 'Save to Template'.

31. Name your file and save it.

Now, we will acquire the data we generated.

32. On the top of the screen, select 'Analysis'

33. In this screen, click 'Select all'

34. On your keyboard, press 'ctrl' and 'c'.

35. Open Microsoft Excel.

36. Press 'ctrl' and 'v'.

Now you have quantified the fluorescent signal.

For exporting the image from Multispectral Viewer, click Export main image.

Select a location to save your image and make sure you change the file extension to .bmp.

Open your image using a picture viewer program.

Exercises

1. Repeat signal quantification for far-red unmixed signal.

2. Try unmixing the B16-GFP mice. Here, paw and bladder give background signals that need to be included as probe in addition to fur. Gut does not give background, which interferes at this wavelenth range. Quantify the specific signal.

3. Export unmixed image from B16-GFP mouse.

Starting files are under /Desktop/Workshop/Experiment5