Illumination Reference Files for the In-Vivo & IS4000 Imaging Systems

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Theory

To accurately measure multiple samples in a single image it is necessary to correct for unavoidable variations in the uniformity of the excitation illumination field across the field of view of the image.

Variations across the imaging field of view arise from:

- Excitation Nonuniformity
- Detection Nonuniformity

When do we need Illumination Reference files?

• For High Resolution X-ray Images

- Corrects for Detector Nonuniformity (CCD & confocal lens)

To make comparisons between fluorescent samples across a field of view

- Corrects for Detector & Excitation Nonuniformities

- Illumination reference files are specific to: Illumination source (type of capture), X-ray filter (X-ray only), f-stop, FOV, Focal Plane, Excitation filter, Emission Filter. If one of these parameters is altered, a new illumination reference must be taken
- Illumination reference files are not specific to exposure time or binning.

Nonuniformity Detailed

1	2	3
4	5	δ
7	8	9

Fluorescence Illumination Reference File

2210 3500 2373 2983 4280 2921 2358 3150 1852	1	2	3	4	5	6	7	8	9
	2210	3500	2373	2983	4280	2921	2358	3150	1852

X-ray Illumination Reference File



1	2	3	4	5	6	7	8	9
15236	18854	18258	16293	19407	19502	13711	16598	16153

Significant differences seen in the mean intensity per image region in both fluorescence & X-ray captures...it is this nonuniformity that must be corrected for quantitative/qualitative comparisons!!!

How to Make an Illumination Reference: Fluorescence

- 1) Establish your fluorescent capture settings and then select illumination reference in the exposure type:
- 2) Place your Epi Field Flattening screen into your system covering the platen completely



How to Make an Illumination Reference: Fluorescence (cont'd)

3) Select Exposure time and binning.

Note: Binning will always be 4x4 unless 8x8 binning is used in capture setting. Exposure time needed varies by emission filter due to efficiency differences of the epi field flattening screen

175W		400W			
Em Filter	Exp time(s)	Em Filter	Exp time(s		
480	0.3	480	0.2		
535	1	535	0.5		
600	1.5	600	1		
670	3	670	2		
700	5	700	3		
750	10	750	5		
790	60	790	30		
830	180	830	60		

securitys. Joron	NIRF 5 mice	New	Preview
User Name: Annotation:		✓ Save ✓ Delete	Expose
Illumination Reference			Protocols
Exposure Time : 30	Sec 👻 X Binning: 4 p	pixels 💌	RGB-1 mouse
	Y Binning: 4 p	vixels 👻	Execute Selected Protoco
			Create/Edit Protocols
pply Reference File : None	🗾 X-Ray	Filter : 0.8 mm 👻	
pply Reference File : None Set Camera To f-stop :	X-Ray	Filter : 0.8 mm 👻	RFID Scans
pply Reference File: None Set Camera To f-stop: 0 2.8 4 FOV:	×-Ray 5.6 8 11 16 22 32 C	Filter: 0.8 mm 💌	-RFID Scans
pply Reference File None Set Camera To f-stop: O 2.8 4 FOV: (mm) R R R	▼ X-Ray 5.6 8 11 16 22 32 C 9 8 9 2 1 9 9 8 8	Filter: 0.8 mm 💌	RFID Scans
pply Reference File : None Set Camera To f-stop : 0 2.8 4 FDV: (mm) R R R Focal Plane : (mm) -5 0 5	5.6 8 11 16 22 32 C 9 8 9 2 2 9 9 8 8 10 15 20 25 30	Filter: 0.8 mm 💌	RFID Scans Apply to Image Clear
pply Reference File : None Set Camera To f-stop : 2.8 4 FOV: (mm) R R R Focal Plane : Excitation Filter : 730	5.6 8 11 16 22 32 C 3 8 8 1 16 22 32 C 3 8 8 8 1 1 16 22 32 C 4 9 1 1 16 22 32 C 5 10 15 20 25 30	Filter: 0.8 mm 💌 2.80 179.60 Tray 💌	RFID Scans Apply to Image Clear

4) Press Expose Button

5) Do not "save" capture setting file

How to apply an Illumination Reference File: Fluorescence

Automatic Application to future captures

After you have successfully captured your illumination reference file:

Select Apply Reference file -> Auto Select

If the reference file is currently open, it will disappear from the screen, indicating a proper application

Images will automatically be processed with appropriate reference file if no error message occurs

c

-Edit

-Edit

160

179.60

Tray 👻

Apply to image

Clear

Done

WL 12 8EOV120EP0 00520790

fwLf2.8F0V120FP0.00570790

MWL (2 8EOV120EP0 00620790

W/Lf2.8F0V120FP0.00650790

WLI2.8FOV120FP0.00670750

MWL(2.8F0V120FP0.00670790 MWLf2.8F0V120FP0.00690790 JWL F2 8EOV120EP0 00720790 7MWLf2.8F0V120FP0.00760830

Excitation Filter

Emission Filter: 790

Camera Temperature: -29.0 Serial Number: 5124

Image Math n-Vivo FX PRO Task -Type: SPO-NIRF 5 mice Settings: -New Preview --Save Calculate Illumination correction User Name: Expos Delete Annotation: NIRF mice corrected Output Image Z Standard Exposure • Protocols RGB-1 mouse Exposure Time: 30.000 Sec 👻 X Binning: 4 pixels -Description: Illumination correct experimental image X with illumination reference Execute Selected Protocol image Y Y Binning: 4 pixels 👻 No. Exposures: Create/Edit Protocols Predict ... Export Options : Final Accumulation Input Image X: Input Image Y - Illumination Constants Illumination Source : Multi-wavelength 💌 Mag. Stage : 🦵 KVP : </u> 35 Preferences -Apply Reference File: None X-Ray Filter : 0.8 mm None **BFID Scans** Set Camera To --2.80 MWL (2 8EOV100EP0 00650700 MWLf2.8F0V120FP0.00460790

Application to a pre-existing capture

Open image to be corrected and the proper reference file

Navigation panel -> Image -> Image Math

Model after below screen shot

-

Replace illegal values with: 0

2011-02-09 17-17-14 -

Cancel

MWLf2.8FOV180FP1 -

Ready to calculate.

OK

C:

Results of Illumination Reference File Application: Fluorescence

No Reference File Applied



Significant Signal Quantification error can occur without illumination reference

How to Make an Illumination Reference: X-ray

n-Vivo FX PRO Settings: SPO-XRY 5 mice New	Preview	1) Create desired captured setting
User Name: Save Save Delete	Expose	2) Select Illumination Reference
Standard Exposure Standard Exposure Time Lapse Exposure Progressive Exposure Radio Isotope Illumination Reference Export Options : Final Accumulation	Protocols RGB-1 mouse Execute Selected Protocol Create/Edit Protocols	 Select Reference File Settings. Always 4x4 binning. Exposure time varies depending on X- ray filter used
Illumination Source: X-Ray Mag. Stage: KVP: 35 Apply Reference File: None X-Ray Filter: 0.4 mm	Preferences	None = 10s exposure
Set Camera To f-stop: 0 2.8 4 5.6 8 11 16 22 32 C FOV: FOV: FOV: FOV: FOV: FOV: FOV: FOUL F	RFID Scans	0.1 mm = 15s exposure 0.2 mm = 20s exposure 0.4 mm = 30s exposure 0.8 mm = 30s exposure
(mm) Recal Plane: (mm) -5 0 5 10 15 20 25 30 (mm)	Clear	4) Remove all samples from image station
Excitation Filter: 0		5) Expose
Emission Filter : 0 Edit Camera Temperature: -29.0 Serial Number: 5124	Done	6) Do not "save" to capture setting file

How to apply an Illumination Reference File: X-ray

Refer to "How to apply an Illumination Reference File: Fluorescence" slide. It is the same!!!

Results of Illumination Reference File Application: X-ray

No Reference File Applied







- Reference file application smoothes image and removes low-signal corners
- Reference files essential for X-ray density conversion and bone density analysis

Thank You!!