Carestream
Molecular Imaging

A division of Carestream HEALTH

Refining epifluorescence imaging and analysis with automated multiple-band flat-field correction

The new KODAK In-Vivo Multispectral Imaging System FX uses an innovative calibration tool, the Kodak Epi Field Flattening Screen, which provides efficient calibration over a wide range of excitation and emission filter combinations. When used with Kodak MI v5.0, software that automates collection, calibration and analysis of multimodal images both *in vivo* and *in vitro*, the screen enables accurate fluorescence measurement of samples placed anywhere on the imaging surface, improving quantitative accuracy and overall throughput.

In both *in vivo* and *in vitro* fluorescence imaging with the Kodak In-Vivo Multispectral Imaging System FX (http://www.carestreamhealth.com/go/molecular), it is often desirable to image multiple animals or samples over a wide field of view, either to improve throughput or to facilitate comparison of experimental sample versus control. To accurately measure multiple samples in a single image, however, it is necessary to correct for unavoidable variations in the uniformity of the excitation illumination field across the field of view of the image. Collectively, the spatial variation of the imaging system is caused by a combination of excitation nonuniformity and detection nonuniformity.

We have developed a technique to correct illumination variation by the use of a new calibration tool, the Kodak Epi Field Flattening Screen, designed to deliver an efficient fluorescence return through a wide range of excitation-wavelength (390–770 nm) and emission-wavelength (440–830 nm) combinations. The fluorescence efficiency of the screen is sufficiently high that most permitted excitation/emission filter pairs only require a 10-second exposure to obtain a calibration image of adequate signal-to-noise ratio to ensure an effective calibration. Software automation using Kodak MI v5.0 makes it possible to collect and archive a library of illumination reference images for flat-field correction over all permitted excitation/emission filter pairs. Later, users can define 'protocols' that automate both the capture of multiband fluorescence images (as well as images taken in other modalities) and the application of the appropriate flat-field correction from the illumination reference library.

In vivo imaging with illumination correction

To demonstrate the value of this method, we conducted the following experiment using a Kodak In-Vivo Multispectral Imaging System FX.

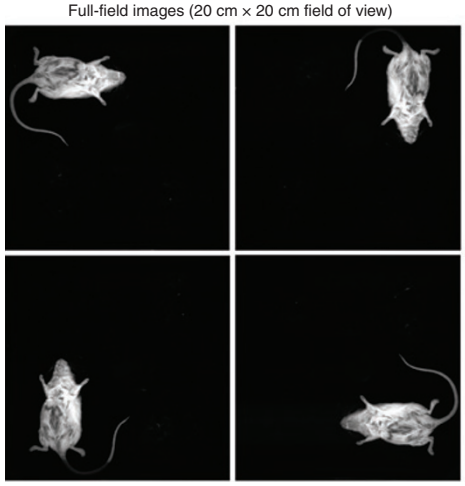

Figure 1 | Kodak In-Vivo Multispectral Imaging System FX white-light reflectance images showing the position of the mouse for each imaging session. In each session, the white-light image was automatically captured along with an X-ray absorption image and 18 epi-illuminated images of optical fluorescence (790 nm emission, multiple excitation wavelengths).

We euthanized a Swiss albino mouse having fur and injected it intramuscularly with Kodak X-SIGHT 640 Large Stokes Shift Dye (http://www.carestreamhealth.com/go/x-sight) and a competitor's dye with a slightly different excitation spectrum (40 pmol each). We placed the mouse in an In-Vivo Animal Imaging Chamber and imaged it four separate times, rotating the chamber (and mouse) 90° with each execution of the imaging protocol (**Fig. 1**). By rotating the chamber, we minimized the effects of repeatedly lifting and repositioning the mouse's extremities with each imaging session. At each rotation the automated image capture protocol collected 18 fluorescence images (using excitation wavelengths from 400 to 730 nm paired with a 790-nm emission filter), one white-light reflectance image and one X-ray absorption image

Douglas Wood, Gilbert Feke, Douglas Vizard & Rao Papineni

Carestream Molecular Imaging, 4 Science Park, New Haven, Connecticut 06511, USA. Correspondence should be addressed to D.W. (douglas.wood@carestreamhealth.com) or G.F. (gilbert.feke@carestreamhealth.com).

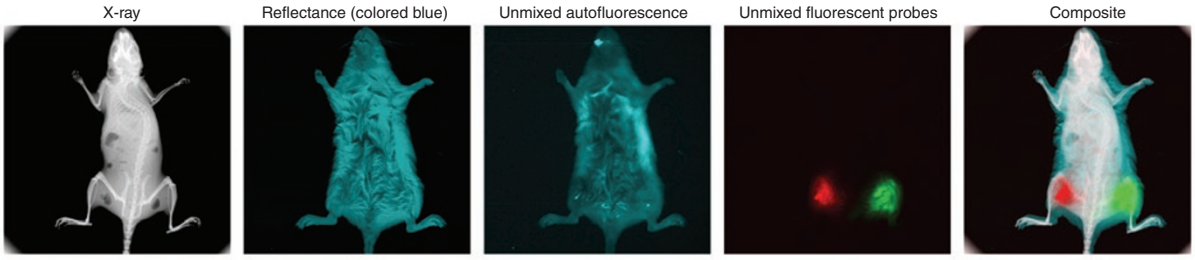

Figure 2 | Multimodal/multispectral images of the mouse produced with Kodak Multispectral Software. The two panels on the left are captured images, and the panels labeled "unmixed" were calculated from a numerical model fit to the flat-field corrected epi-illumination image cube. The last panel is a composite of all except the unmixed autofluorescence image.

(using the Kodak Radiographic Phosphor Screen). All the images were automatically flat-field corrected using the previously captured calibration images made with the Kodak Epi Field Flattening Screen or the Kodak Radiographic Phosphor Screen.

Improved fluorescence intensity measurements

With automatic flat-field correction, we found only a 3% variance of the intensity in the fluorescence images taken with the mouse in the four different locations on the imaging surface. This was a dramatic improvement compared to the 14% variance that we measured when flat-field correction was not applied.

Next we created numerical models of the absorption curves of the two fluorescent probes and the background autofluorescence, and used these models in a nonlinear least-squares fit using Kodak Multispectral Software to 'unmix' the three spectral signatures that contribute to the measured excitation spectrum at each pixel (**Fig. 2**). Our analysis clearly distinguished the two probes (even though their excitation peaks are only 20 nm apart), and the imaging system registered the fluorescence images with images taken in other modalities. Note that the unmixed autofluorescence closely matched the reflectance image, as expected for this fur-covered mouse.

To quantitatively test the accuracy of combining the flat-field correction and the spectral unmixing analysis, we compared the unmixed probe images taken in each quadrant (after rotating all of the images to the same orientation; **Fig. 3**). We made two comparisons. First, we observed that the detailed morphology seen in all of the unmixed images was very similar, as should be the case considering that we did not reposition the mouse with each rotation. Without the flat-field correction, the images did not show this high degree of similarity. Second, we compared the measured intensities of the unmixed probes and found a variation of only a few percent. We observed much larger variation if flat-field correction was not applied.

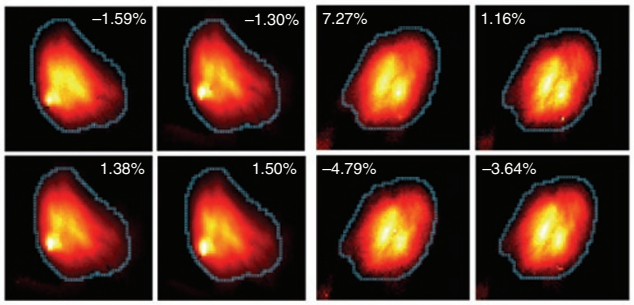

Figure 3 | Unmixed images produced with Kodak Multispectral Software. To facilitate comparison, the images of each dye that were captured with the mouse in the four different positions on the imaging surface have been rotated to the same orientation. The same region of interest (ROI) was used to measure all of the features in a given panel. Values in images show the percent deviation of the net intensities of the probe emission for each ROI from the mean of all ROIs in a given panel.

Conclusions

Using the new Kodak Epi Field Flattening Screen and the Kodak In-Vivo Multispectral Imaging System FX, we demonstrated that when variation of the excitation illumination field of epifluorescence images is measured and corrected, the spectrally unmixed signals of specimens (subjects) can be reliably compared with an accuracy of a few percent, even when the samples are distributed across the entire 20 cm × 20 cm field of view. Additionally, these corrections can be easily incorporated into an automated data collection system that captures flat-field corrected images in multiple modalities.