Tissue autofluorescence: watch out what's on the menu…

Older readers may remember a television advertisement from the 1970-1980s for Ready Brek: following a hearty breakfast from this brand of porridge, children were surrounded by a warm glow all day (see Figure 1 and http://www.youtube.com/watch?v=i1KUoS3mmvM or http://www.youtube.com/watch?feature=endscreen&NR=1&v=6c3n-mh5bck

Figure 1: Warm memories: This classic Ready Brek advertisement from the 1970s shows children going off to school - fuelled by their Ready Brek breakfast and seeming to 'glow' in the darkness of a cold winter's morning.

Well, it looks like mice and rats have been doing something similar for many years, without having to eat this somewhat taste-free cereal preparation (authors' personal opinion!). Their highly optimised diet seems to provide just such a glow when *in vivo* fluorescence imaging is attempted in the deep red part of the optical spectrum. During such imaging, any unwanted fluorescence (e.g. tissue autofluorescence) is a significant limiting factor that can degrade contrast, sensitivity and resolution. Autofluorescence is defined as the emission of light by biological structures following absorption at a lower wavelength (see e.g. Gallas and Eisner 1987, Andersson *et al.* 1998). When exciting a sample in the ultraviolet or visible wavelength ranges, such fluorescence can affect the ability to detect signals produced by intentionally added fluorophores. One way to overcome this difficulty is to use excitation and emission wavelengths in the far red – near infrared, where tissue autofluorescence is significantly lower.

Although operation at >600 nm is commonly exploited when working with cell preparations or with thin tissue sections, life is not quite so straightforward when attempting live animal fluorescence imaging, as we found to our cost! Our group had developed a fluorescence image guided surgery instrument which could operate in the near-infrared (~780 nm) and the deep red (~660 nm), the latter aimed at exploiting fluorescence from Cy5 dye and its variants. We postulated that the use of 660 nm excitation would yield good results, since background fluorescence would be low. To our surprise, the mouse glowed brightly even before any dye was injected (Figure 2).

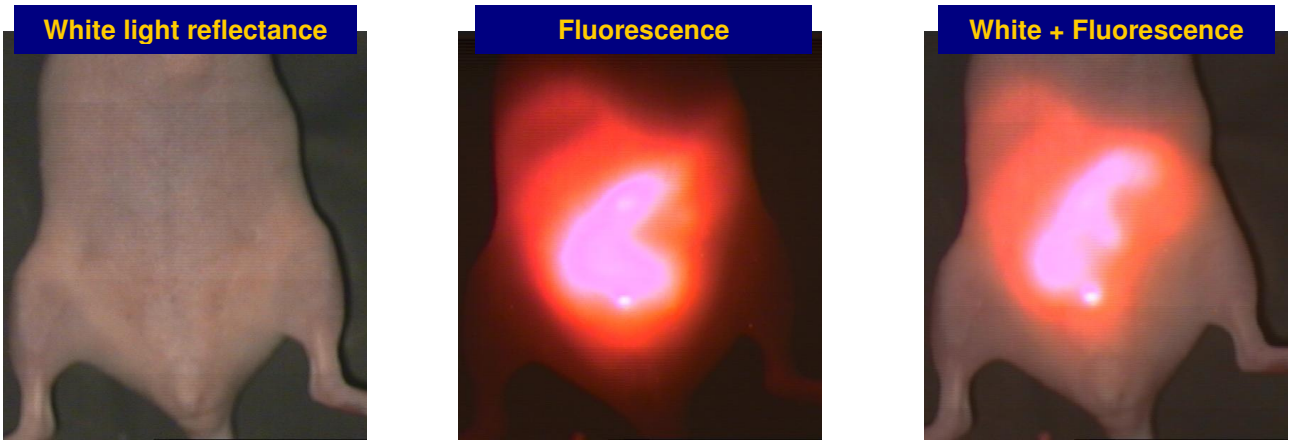

Figure 2: Left: white light reflectance image of a nude mouse. Middle: autofluorescence from the bowel of the same mouse, excitation 660 nm, emission 665-775 nm (40 ms integration time, f/2.8 optics, ~10 mW/cm^2 excitation power density). Right: white light reflectance and simultaneous fluorescence image. A similar result was found in hairy mice.

This “autofluorescence” was so strong that the image signal saturated even at the system’s lowest sensitivity. It was obvious from Figure 2 that the gut and bowels were somehow involved and this was confirmed following surgery: the source of the fluorescence contamination was likely to be associated with food (Figure 3).

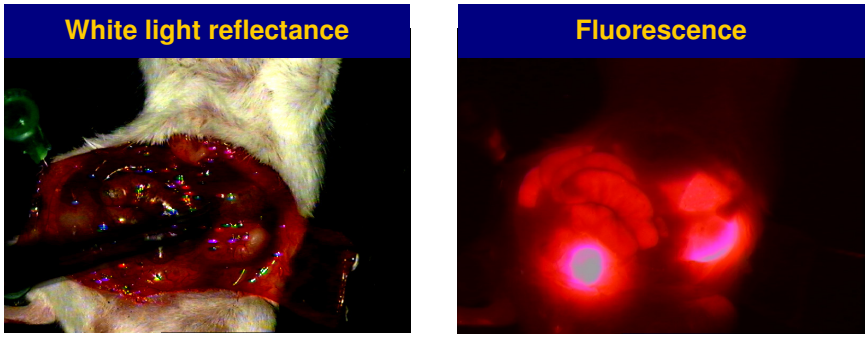

Figure 3: Autofluorescence from internal organs (40 ms integration, f/2.8 optics, ~10 mW/cm^2 excitation power density).

We therefore obtained a sample from the diet used to feed the mice: SDS RM3 (E) DU diet, www.sdsdiets.com) and imaged it with our fluorescence system. As expected, the single pellet of food was strongly fluorescent when excited with 660 nm light (Figure 4). Excitation at the higher wavelength (785 nm) did not show significant emission.

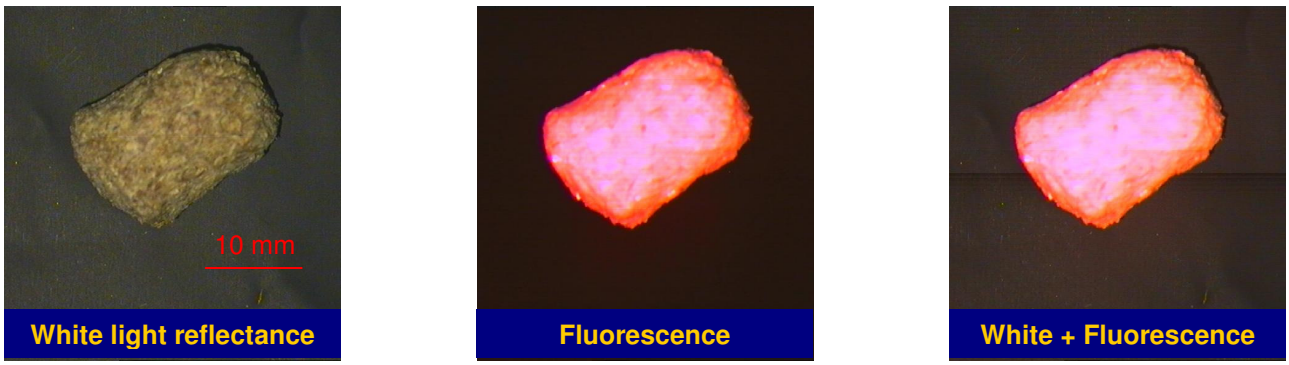

Figure 4: SDS RM3 (E) DU diet when excited with 660 nm light (40 ms integration, f/2.8 optics, ~10 mW/cm^2 excitation power density). Excitation at longer wavelength did not show significant fluorescence.

In order to measure the fluorescence excitation and emission spectra of the diet, a single pellet was broken up and placed in a beaker containing ~5 ml ethanol. The mixture was stirred at room temperature for 2-3 hours and then centrifuged at 10000 rpm for 200 sec to remove the solid material. The liquid component was extracted and its fluorescence properties measured using a spectro-fluorometer (Perkin Elmer LS50B). The resulting spectra are shown in Figure 5.

It has been recently reported that chlorophyll from the alfalfa in standard mouse food is fluorescent in the far red – near infrared wavelengths (Troy *et al.* 2004). Alfalfa is a perennial flowering plant and a source of vitamin D. Mice diets with a high content of vitamin D are likely to be rich in alfalfa, and therefore are not optimal for animal experiments which involve imaging at these wavelengths. While spectral unmixing methods can be used to minimise the consequences of this unwanted signal (Mansfield *et. al*, 2005), it is much simpler to remove the ‘glow’ altogether.

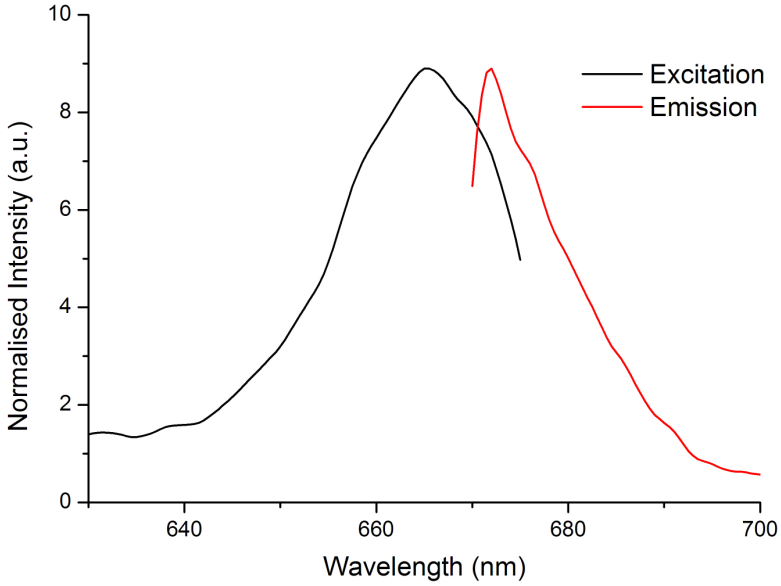

Figure 5: Excitation and emission spectra of SDS RM3 (E) DU mice diet in ethanol solution (normalised values). Results show excitation and emission peaked in the far red region. This suggests that excitation above 690-700 nm or below 630 nm is probably acceptable.

In order to improve the contrast and sensitivity of fluorescence detection without using any sort of spectral unmixing technique, we purchased an alfalfa-free diet (Harlan 2919, www.harlan.com). Figure 6 shows a comparison between the two diets in terms of fluorescence intensity. Clearly, the use of such a diet overcomes most of the problems.

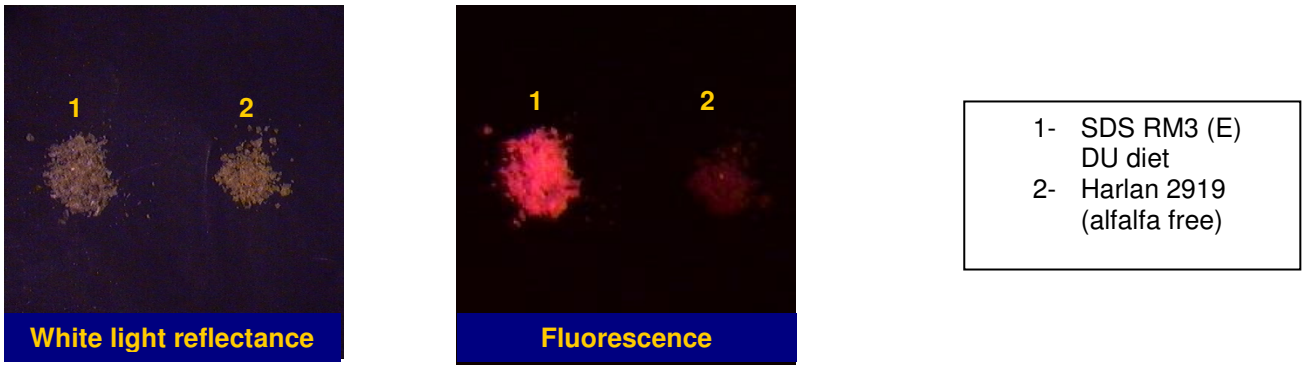

Figure 6: Comparison of rodent diets with and without alfalfa, when excited with 660 nm. The fluorescence from the SDS RM3 (E) DU diet was significantly higher than that from the Harlan 2919 diet. The integration time was 320 ms and f/2.8 optics were used as before, but the excitation power density was reduced to ~1 mW/cm^2 to ensure that the imager was not overloaded by the brighter sample.

Nude mice were fed with the new alfalfa-free diet for 11 days and imaged. Figure 7 shows the fluorescence emission from internal organs at 660 nm excitation. Note that this image was obtained using the same camera settings and excitation source as in Figure 3. Although some autofluorescence is still present, its intensity is substantially lower, with no saturation of the image signal. As a result, a higher signal-to-background ratio can be achieved.

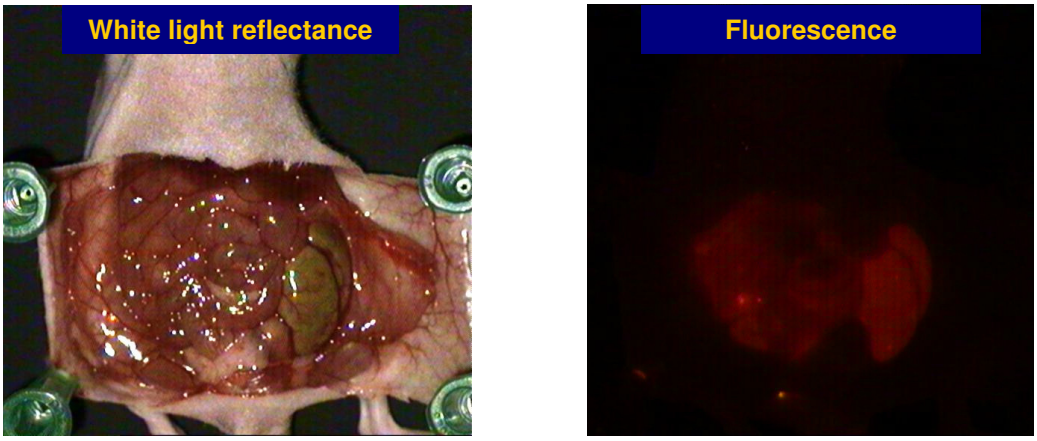

Figure 7: Autofluorescence from internal organs of nude mice fed with alfalfa-free diet (40 ms integration, f/2.8 optics, ~10 mW/cm^2 excitation power density).

In conclusion, we presented a brief description of how a standard diet to feed mice can influence the signal-to-background during *in-vivo* fluorescence imaging. Prior imaging, one should check the fluorescence level of the diet, particularly when exciting in the far red wavelengths. An inappropriate diet could compromise the results of an experiment.

References

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These measurements presented here were performed in July 2012 by Davide Volpi. This note was prepared by D Volpi and B Vojnovic in August 2012, with the intention of alerting potential users of in-vivo fluorescence imaging methods to the sort of pitfalls which we experienced. We are very grateful for the assistance of A. Ahmed, C. Becker and M. El-Kasti (Nuffield Department of Obstetrics and Gynaecology, Oxford) who performed animal surgery.

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