Hyperspectral in-vivo two-photon microscopy of intrinsic fluorophores

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Abstract: In-vivo two-photon imaging of intrinsic fluorescence allows metabolic function to be evaluated on a cellular level. A method of validating, identifying and separating the fluorophores present in an in-vivo two-photon image is described.

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1. Introduction
Two-photon microscopy of in-vivo intact tissues allows detailed and high-resolution optical imaging of tissue structure and function. While a wide range of exogenous contrast agents are becoming increasingly available, it is also of significant interest to study sources of intrinsic fluorescence such as NADH, FAD and collagen[1]. These fluorophores can serve as intrinsic biomarkers of metabolism, or of native structure, and allow imaging without the need to introduce artificial compounds which may perturb normal function. Imaging intrinsic fluorophores reliably on microscopic length-scales in many different tissues (e.g. heart, brain and skin) could allow unprecedented investigations of tissue function on a cellular scale.

Microscopy of intrinsic fluorophores is challenging for two reasons: First because signals can be quite weak compared to exogenous contrast agents. Second, it can be difficult to categorically identify which fluorophore is being visualized. NADH has been notoriously difficult to isolate in-vivo partly because of its very low excitation wavelength (355nm) and also because of overlap of its emission with other fluorophores such as collagen. A recent study discriminated between bound and unbound NADH via its characteristic fluorescence lifetime, however relying on lifetime can be costly, complex and slow [2].

In this paper we demonstrate a new approach to two-photon microscopy, which can readily delineate multiple unknown fluorophores in-vivo. We borrow spectral unmixing techniques from wide-field small animal imaging, and apply them to excitation-scanned two-photon microscopy data (acquired using our fully-automated home-built in-vivo imaging system). We demonstrate the method on in-vivo intrinsic fluorescence images of skin on a nude mouse ear.

1. Instrumentation
Figure 1 shows a schematic of our recently completed two-photon microscope system, optimized for in-vivo imaging. The system uses a Spectra Physics MaiTai XF broadband Ti:Sapphire laser, and galvanometers from Cambridge Technologies. The system has three photomultiplier tube detectors with fully interchangeable dichroics and emission filters. All motion is motorized: the X and Y translation stages and the objective’s Z-translator. The laser is automatically attenuated using a motorized rotation stage holding a half-wave plate. Our software is a graphical user interface written in Matlab. It controls all motion, detector channel amplifier gain and bandwidth settings, image acquisition, image display and online processing. The software has also been written to incorporate control of the laser. This provides the opportunity to synchronize data acquisition with tuning to different excitation wavelengths, while also monitoring laser power levels and stability.

Figure 1. Our home-built two-photon microscope for in-vivo imaging.
2. In-vivo imaging of intrinsic fluorophores

Figure 2 (top) shows an X-Y two-photon image acquired using our home-built system. The image was acquired in-vivo from the ear of an anesthetized nude mouse using 800nm excitation, and an emission band between 350nm and 505nm. No exogenous contrast was added prior to imaging. Clear intrinsic contrast is visible. The image at the bottom of Figure 2 shows an X-Z cross section of the region indicated between the two dotted lines in the upper X-Y image (acquired as an image stack at 2 micron steps in Z). The depth-location of the X-Y image is indicated by the single dotted line in the lower X-Z image. This shows that the X-Y slice is on a slight angle relative to the upper surface of the skin, transecting the very bottom of the epidermis on the left side and cutting into the deeper dermis on the right. The circular white dots in the X-Y image are fine hairs, which can also be seen in cross section in the X-Z image. While acquiring such clear images of intrinsic contrast with in-vivo two-photon microscopy is potentially very useful, it is difficult to interpret the image fully. Although we were imaging with two other emission channels (505-560nm and 560-700nm), the majority of the detected signal was in the first <505nm channel. An experiment where multiple dyes are required would deliberately separate those dyes to give good contrast in standard emission channels. However, this luxury is not available for intrinsic signal imaging. It is clear that a method is needed to allow improved, quantitative analysis of these potentially highly useful images.

3. Hyper-spectral excitation wavelength imaging

We acquired an ‘excitation wavelength scan’ of the same area as shown in Figure 2. This scan adjusted the laser wavelength through from 710nm to 920nm in 5nm steps and acquiring three 400x400 pixel images at each wavelength. The overall scan took 3 minutes to complete (which could be reduced to be limited only by the speed of the laser tuning). The laser power was monitored both as reported by the laser, and via a laser diode monitoring a reflected beam. The wavelength-dependent attenuation of our excitation-path optics was also measured and accounted for. Each successive wavelength scan was normalized by the square of this wavelength-dependent laser power variation. Measurements were also repeated tuning the laser backwards from 920nm to 710nm and results were highly repeatable. The result of the scan was a cube of imaging data where, for each detector channel, each pixel represents a detailed excitation spectrum of the tissue at that location.

The information content of these spectra can be exploited in many ways [7, 8]. We began by selecting five ‘seed’ regions from the series of images - selecting X-Y areas where interesting and distinctive structures appeared to be present in the different wavelength-images. These seed regions are shown as small color boxes in Figure 2. We then performed a non-negative least squares fit to the entire data set, to extract all of the pixels with similar excitation spectra to our ‘seed’ areas. The ‘basis’ spectra extracted from the raw data are shown in the top right of Figure 3. The ‘basis’ images corresponding to each sampled spectrum are shown in the left-hand column of Figure 3. Two combinations of color-coded merges of these images are shown to the right.

In the first (spectrum 1) image, we see an ordered structure arranged in a pattern from the top left to the lower right of the image. The spectral characteristics of this structure are large absorption peaks at 830nm, 885nm and 900nm. The ‘spectrum 2’ image, while having fairly similar spectral features to spectrum 1, shows an almost orthogonal structural arrangement. The ‘spectrum 3’ image clearly highlights the white dots which represent cross-sections through hairs. The spectrum has a large absorption at 745nm which may represent keratin autofluorescence. Spectrum 4 clearly reveals sebaceous glands around the lower parts of the hair follicles (recall from Figure 2 that the right side of this image-plane is deeper into the dermis than the left side). The spectrum of these glands contains excitation peaks at 745nm, and then at around 833nm and 853nm. The ‘spectrum 5’ image shows the circular regions around each hair, representing the hair follicle. It also shows signal more from the epidermis (left side) of the image (in contrast to the ‘spectrum 4’ image which highlights the deeper dermis on the right side of the image). The major differences between spectrum 4 and spectrum 5 are the higher absorption of spectrum 5 at 730-750nm, and that spectrum 5 is lacking the peak at 853nm.

It is clear that this analysis has yielded a dramatic increase in the information available from our intrinsic signal images. We have clearly delineated five separate structural components of this single layer of skin. Further, we have isolated the characteristic excitation spectra of each of these structural components, offering the ability to directly

![Figure 2. Two-photon intrinsic signal image of a nude mouse ear at 800nm excitation. (top) X-Y view, (bottom) X-Z view between dotted lines in X-Y view. (dotted line in X-Z view shows depth-plane of X-Y view). Colored boxes indicate ‘seed regions’.

Figure 3. Hyper-spectral excitation wavelength imaging image stack at 2 micron steps in Z). The depth-location of the X-Y image is indicated by the single dotted line in the lower X-Z image.
identify the chemical composition of each of these structural components by isolating the chemical constituents responsible for each specific excitation peak. To date, there do not appear to have been any comprehensive studies of the two-photon excitation spectra of intrinsic fluorophores (particularly in-vivo). Various publications have shown partial two-photon excitation spectra for NADH [3, 4] and keratin [5], yet many other intrinsic fluorophores are likely to be contributing such as elastin, collagen, NADH, flavoproteins and porphyrins. It has been documented that these may have several sub-types with different spectral properties. There are also likely to be contributions from second harmonic generation from both collagen and elastin [6]. Further work will be required to properly characterize each component of the in-vivo excitation spectrum of tissues to allow more routine analysis and segmentation of hyperspectral excitation-wavelength scans. This work can readily be achieved using our method.

5. Summary

We have presented a new method to acquire and process two-photon images which exploits the differences in the excitation spectra of constituent fluorophores. This could be a particularly useful tool for characterization of intrinsic fluorophores where the number and type of fluorescent (or second harmonic generating) species present is unknown, and/or emission spectra overlap substantially. A significant benefit of this method is that it provides both contrast enhancement, and a set of representative excitation spectra for each component which can be compared to known spectra of intrinsic fluorophores. We will present our latest progress in characterization of intrinsic fluorophores and in the development of analysis methods for in-vivo two-photon microscopy.

6. References


Figure 3. Hyperspectral separation of two-photon intrinsic fluorescence excitation-scan images (nude mouse ear in-vivo). (left) spectral component images derived from excitation scan where spectra (top right) were extracted from ‘seed’ regions shown in figure 2 using a non-negative least squares fit. Right shows merges of (top) spectral components 1, 2 and 3 colored red, green and blue, and (bottom) components 3, 4 and 5, colored blue, yellow and cyan.