Enhanced detection of tissue auto-fluorescence by one-photon ultrafast pulsed illumination

Molecular fluorescence[1,2] has revolutionized qualitative as well as quantitative analytical detection techniques with wide-ranging applications, particularly those rendered through darkfield fluorescence microscopy of live cells[3]. For nearly a century, fluorescence microscopy has been used in clinical studies[4]. Tissue autofluorescence from endogenous fluorophores has been shown to be quite useful in understanding cellular metabolism, obviating the need for histological staining or expressing specific genes for fluorescent proteins. However, the poor absorption coefficient and low fluorescence quantum yield of endogenous fluorophores have limited the wide application of fluorescence microscopy with tissue autofluorescence as a diagnostic tool. In an earlier work on fluorescence enhancement via pulsed excitation, we have shown how femtosecond pulsed excitation can lead to huge fluorescence enhancement[5]. Here, we extend our previous work on cervical cancer detection by quantitative two-photon autofluorescence detection[6]. We qualitatively show how one-photon autofluorescence yield can be enhanced by femtosecond pulsed excitation instead of conventional continuous-wave (CW) excitation. Due to the enhanced autofluorescence, we can record breast-tissue micrographs from patients suffering from breast cancer, which is the most common form of cancer in India[7].

A commercially available confocal microscope system (FV300 scan-head coupled with IX71 inverted microscope, Olympus Inc., Japan) was used for imaging. Second-harmonic of the fundamental centred on 780 nm from a commercial femtosecond laser (MIRA-900F producing ~120 fs pulses at 76 MHz repetition-rate and pumped by VERDI-5, Coherent Inc., California, USA) was used as excitation. Thin sections (20 μm) of fresh tissue samples from normal and abnormal biopsies of ten patients, cut using standard tissue-cutting machine (Leica Inc.), were placed on microscope slides and carefully covered with a cover slip to avoid retaining any air-bubble. Images were collected using FLUOVIEW software (Olympus Inc.). The average power after the objective lens (×40, oil-immersion) was ~0.1 mW. Fluorescence was measured using a 510 nm cut-off filter.

Autofluorescence from cancer cells, including breast cell lines has been studied by other researchers as well[8,9]. In all these studies, low-power CW laser excitation (or UV-VIS lamp excitation) was used. The average to peak power ratio of a ~100 fs pulsed excitation at 76 MHz repetition-rate is about $1 : 10^5$. This huge instantaneous photon flux from femtosecond laser pulses can be successfully used to circumvent the low one-photon absorption cross-section (as well as low autofluorescence quantum yield). Ultrafast (0.1–1 ps) pulsed excitation at high repetition-rate (10–100 MHz) is routinely used in two-photon fluorescence microscopy owing to the low two-photon absorption cross-section of common fluorophores. Also, the radiative lifetime nicely fits into the 10–100 ns time-lag between the consecutive pulses[10]. However, considering one-photon fluorescence, commercial fluorescence microscope systems mostly use CW illumination; pulsed excitation is used to probe and control fluorescence dynamics either for contrast enhancement (e.g. by lifetime distribution measurement in FLIM microscopy) or for resolution enhancement (e.g. in STED microscopy), but not for overall fluorescence yield enhancement. Earlier, we had demonstrated significant *in vitro* fluorescence enhancement with ~100 fs pulsed excitation at 76 MHz repetition-rate[5]. Here we show an *in vivo* application with endogenous fluorophores with quite low brightness (i.e. product of absorption cross-section and fluorescence quantum yield).

Figure 1 shows fluorescence images of normal breast tissue samples from four different patients. Similar images of malignant breast tissue samples from the same patients are shown in Figure 2. In spite of observable increase in average tissue autofluorescence from a nearly uniform similar region of interest, we could not follow any consistent pattern for this increase. The wide structural and morphological variation of breast tissue samples was too overwhelming to arrive at any definitive conclusion based on quantitative autofluorescence. Also, the limited number of patients restricted any statistical analysis of the data. Nevertheless, it shows the potential of this excitation scheme in sensitive autofluorescence detection.

Among the vast intrinsic tissue fluorophores, flavin adenine dinucleotide (FAD, broad absorption ~400 nm and fluorescence maximum ~530 nm) turns out to primarily contribute to the detected autofluorescence considering

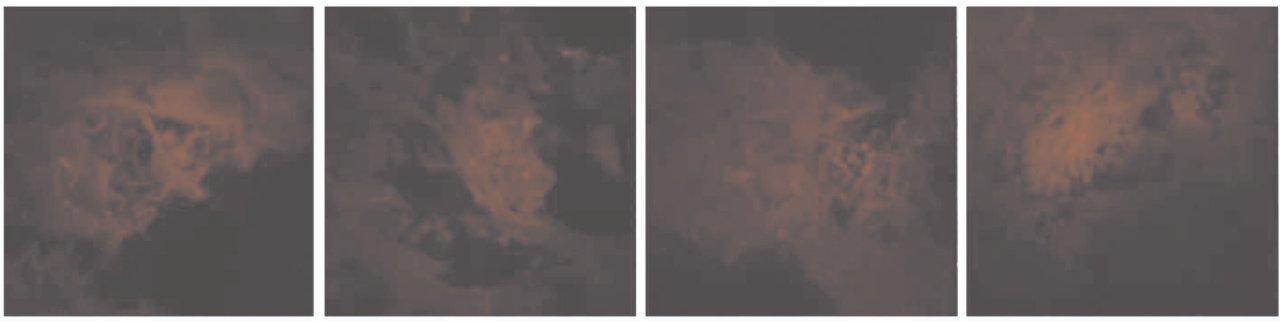

Figure 1. Fluorescence image of normal breast tissue samples from four patients.

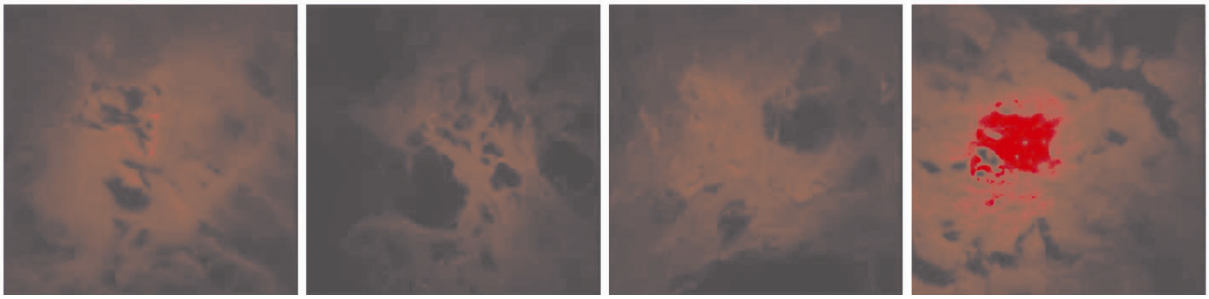

Figure 2. Fluorescence image of malignant breast tissue samples from four patients (the ordering of patients from left to right exactly corresponds to that in Figure 1).

the excitation ~400 nm and emission ≥ 510 nm, and the reduced fluorescence in malignant tissues may be attributed to a reduced FAD level. Note that, contrary to our observation, several earlier studies have reported elevated total integrated autofluorescence intensity in cancerous breast tissue[11,12], which may result from different excitation wavelength (~337 nm) and/or different tissue morphology. Thus, further spectroscopic investigation with larger number of case studies needs to be carried out in order to pinpoint the exact molecular species (FAD and other endogenous fluorophores, e.g. porphyrins) responsible in enhanced/decreased breast tissue autofluorescence.

To summarize, we qualitatively showed how femtosecond-pulsed (one-photon) excitation helps in appreciable autofluorescence generation. Implementing this excitation scheme to quantify autofluorescence from normal and malignant tissues will be the next immediate step for early diagnosis of cancer.

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Ultrastructures in the lateral part of *Nummulites vredenburgi* Prever (Foraminiferida)

The Middle Eocene larger foraminifera *Nummulites vredenburgi* Prever was erected by Vredenburg[1,2] from Kutch, western India. This operculiniform *Nummulites* species was subsequently examined by several workers[3–6]. Previous studies involving the foraminifer were primarily based on light microscope observations. Few scanning electron microscope (SEM) illustrations showing pores and canals in *N. vredenburgi* have been also published[5]. Our collection of several well preserved void dimorphic tests of *N. vredenburgi* from western Kutch has greatly facilitated the examination of test ultrastructures using the SEM. We report here wall modification and cavity development in the lateral part of *N. vredenburgi* and provide a first hand functional analysis of these ultrastructures.

Dimorphic forms of *N. vredenburgi* were collected from a 0.3 m thick foraminiferal shell bed occurring in the upper part of the Middle Eocene Harudi Formation stratotype near Harudi village (23°30′32″N, 68°41′13″E) in western