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N_2 laser excited autofluorescence spectroscopy of formalin-fixed human breast tissue

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Abstract

The paper reports results of an in vitro study on autofluorescence spectroscopy of fresh and formalin-fixed human breast tissue samples to investigate the effect of formalin fixation on the measured autofluorescence spectra. It also explores the applicability of the approach in discriminating cancerous from the uninvolved sites of the formalin-fixed breast tissues based on their autofluorescence spectra. A probability-based diagnostic algorithm, making use of the theory of relevance vector machine (RVM), a powerful recent approach for statistical pattern recognition, was developed for that purpose. The algorithm provided sensitivity values of up to 97% and specificity values of up to 100% towards cancer for both the independent validation data set as well as for the training data set based on leave-one-out cross-validation. These results suggest that autofluorescence spectroscopy may prove to be a valuable additional in vitro diagnostic modality in clinical pathology setting for discriminating cancerous tissue sites from normal sites.

Keywords: Autofluorescence spectroscopy; Ductal carcinoma; Diagnostic algorithm; Formalin-fixed tissue; Histopathology

1. Introduction

In current medical practice, excisional biopsy followed by histology is the gold standard for definitive diagnosis of cancer [1]. The approach makes use of high-resolution image information of the cellular and the sub-cellular structures of properly preprocessed and appropriately stained specimens of tissue kept in the field of view of a microscope. Despite its widespread success, a major drawback of this traditional approach is that the diagnosis requires expert interpretation of the microscopically derived histopathological information that suffers from both human errors as well as sampling errors [1]. This is particularly problematic in the context of diagnosing tumors that are atypical or lack morphological features useful for differential diagnosis. An alternate technique that may help overcome these limitations is the technique based on autofluorescence spectroscopy of human tissues [1–4]. The underlying principle of the approach is that the onset and the progression of a disease like cancer is often accompanied by biochemical and morphological changes that are reflected in the measured autofluorescence spectra of tissue. Considerable work carried out over the last decade has shown that autofluorescence from human tissue can be used for quantitative, in situ, near-real time and non-invasive diagnosis of cancer [2–4]. It is pertinent to note here that this spectroscopic approach may also provide an additional, in vitro diagnostic modality in clinical pathology setting. This may provide to a pathologist a quantitative diagnostic feedback of the disease. However, this necessitates validating the applicability of the method on formalin fixed resected tissue samples that are used for histopathology. This is important because formalin fixation is known to dehydrate the tissue [5] and change its hemoglobin content [6], both of which

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are expected to lead to significant changes on autofluorescence spectra of the tissue. Since most of the earlier in vitro studies [2,3,7–10] were motivated by the objective of establishing the potential of the approach for in situ, non-invasive diagnosis, specific care was taken in these studies to use fresh, unfixed tissue samples to simulate the in vivo condition as close as possible. The concern for tissue handling protocols used in these studies made a few groups investigate autofluorescence from formalin-fixed tissue [6,11]. While Fillipidis et al. [6] reported in vitro autofluorescence spectroscopic studies in peripheral vascular tissues and showed significant differences in measured autofluorescence from fixed and unfixed tissue using visible wavelength excitation, similar studies were reported by Xu et al. [11] in mouse skeletal muscle under two-photon excitation. However, to the best of our knowledge, there are no published reports on applicability of the approach for in vitro diagnosis in formalin-fixed tissues. Even the effect of formalin fixation on the autofluorescence spectra of human breast tissue samples is not documented. The effect of formalin fixation on Raman spectra has received relatively more attention [5]. Whereas changes in Raman spectra due to formalin fixation were significant for human bronchial tissue, for breast tissue, the change was not significant [5].

We report in this paper the results of an autofluorescence spectroscopic study carried out on fresh and formalin-fixed human breast tissue samples. The objective was to investigate the possible effects of formalin fixation on the measured autofluorescence spectra from human breast tissue samples and understand the reasons for the observed spectral differences between fresh and formalin-fixed breast tissue. We also explore, for the first time to our knowledge, the possibility of autofluorescence diagnosis of cancer under formalin fixation condition. We have made use of a diagnostic algorithm developed using the recently formulated theory of relevance vector machine (RVM) [12] for this purpose. The Bayesian formulation of the RVM based algorithm made it possible to provide a quantitative estimate for the confidence with which a tissue site can be classified in a specific group (normal or malignant). The diagnostic results yielded by the algorithm demonstrate that the autofluorescence technique may be developed as a valuable addition to standard histopathological procedures for clinical diagnosis of formalin-fixed tissue specimens.

2. Materials and methods

2.1. Instrumentation

Autofluorescence spectra from the breast tissue samples were recorded at 337 nm excitation using a N_2 laser based portable fluorimeter. A schematic diagram of the fluorimeter is shown in Fig. 1. It comprised of a sealed-off pulsed N_2 laser, a spectrograph (Acton Research Corporation, USA), an optical fiber probe and a gateable intensified CCD detector (4 Quik 05A, Stanford computer optics, Inc, USA). The diagnostic probe, developed in-house, was a fiber bundle, which had two legs; one contained a single quartz fiber (400 μm core diameter, 0.22 NA) and the other contained six quartz fibers (400 μm core diameter, 0.22 NA). The two legs merged to form a common fiber bundle that consisted of a central fiber, surrounded by a circular array of six fibers. The central fiber delivered excitation light to the tissue surface and the six fibers surrounding the central fiber collected tissue fluorescence from the surface area directly illuminated by the excitation light. The proximal ends of the collection fibers were arranged in a vertical array and the light coming from the distal end was imaged at the entrance slit of the spectrograph (Acton Research Corporation, USA) coupled to the intensified CCD detector. All the fibers used had core diameter of 400 μm and NA of 0.22. The common end of the fiber bundle was enclosed in a stainless steel (SS) tube (9 mm outer diameter and 60 mm long). The tip of the probe was shielded by a quartz optical flat 2 mm thick to provide a fixed distance between tissues and the fibers for improved collection of fluorescence and also to protect contamination of the fiber tips with tissue fluids. The spectral data acquisition was computer controlled. The delay between N_2 laser pulse and the intensified CCD camera shutter was adjusted to pick up tissue fluorescence. The gate-width used was 100 ns. The autofluorescence spectra were recorded in the 375–700 nm spectral range with the tip of the fiber-optic probe placed in contact with the tissue surface. The overall spectral resolution of the system was ~2 nm.

In order to monitor the N_2 laser power incident on tissue, the cavity of the N_2 laser used in the setup was formed with a high reflectivity (~99%) mirror and an uncoated quartz window was used as the output coupler. The small N_2 laser output leaking from the rear mirror was used to excite a phosphor material glued to the tip of a quartz fiber. This fiber was coupled to the spectrograph along with the six fluorescence collection fibers and the N_2 laser excited luminescence coupled to the fiber was used to monitor the laser output. The luminescence was verified to scale linearly with the N_2 laser power at the power levels available through the rear mirror. Further, at this level of the N_2 laser radiation no photobleaching of the phosphor material was observed. Since the emission of the phosphor was at ~520 nm, we could use a N_2 laser cut off filter to prevent N_2 laser radiation back scattered from the tissue from reaching the detection system. In absence of this filter considerable distortion of the weak tissue fluorescence was observed particularly in the spectral regions 350–400 nm which overlaps with the first order of N_2 laser radiation and