DEVELOPMENT OF AN *EX VIVO* QUANTITATIVE SPECTROSCOPIC SCANNER

D. S. Ferreira[1], N. Lue[2], G. Minas[1], M. S. Feld[2], K. Badizadegan[3,4] and C. Yu[5]
[1]*Centro Algoritmi, Universidade do Minho, Campus de Azurém, 4800-058, Guimarães, Portugal*
[2]*GR Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology*
77 Massachusetts Avenue, Cambridge, MA 02139, U.S.A.
[3]*Departments of Pathology and Health Sciences and Technology, Harvard Medical School, Boston, MA 02114, U.S.A.*
[4]*James Homer Wright Pathology Laboratories, Massachusetts General Hospital*
WRN219, 55 Fruit St, Boston, MA 02114, U.S.A.
[5]*Optics Research Laboratory, Research & Development Division, Canon U.S.A., Inc.*
9030 South Rita Road, Suite 302, Tucson, AZ 85747, U.S.A.

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Abstract: We describe an *ex vivo* quantitative spectroscopy (QS) scanning platform which enables integration of different optical modalities for the assessment of *ex vivo* tissue properties. As a first implementation, the QS scanner combines diffuse reflectance spectroscopy (DRS) and intrinsic fluorescence spectroscopy (IFS) to provide a multidimensional image of tissue structural and biochemical properties. The wide area coverage is achieved by mechanically scanning of the optical probe. The spectroscopic data is taken one grid at a time with variable grid-to-grid (GTG) distance and field of view (FOV). The *ex vivo* tissue surface under examination can have a variable size since both GTG distance and FOV can be controlled. We demonstrate the clinical utility of this system using an *ex vivo* tissue model with ultimate goal of imaging excised tissue margins.

1 INTRODUCTION

Fast and reliable intra-operative diagnosis is critical for the success of oncological surgery in a variety of organ systems. After any cancer tissue resection it has to be ensured that all malignant tissue was removed and for that a surgical pathologist has to examine the tissue margins (Haka, 2006). Current clinical standards include visual inspection of the tissue, followed by selective assessment of any suspicious sites by frozen sectioning and rapid histological evaluation. This procedure is still not very efficient since according to a study by the College of American Pathologists (Novis, 1997) a significant number of hospitals do not routinely provide intra-operative feedback to the surgeon within 20 minutes of tissue delivery, adding costs and an increased risk of morbidity associated with extra time spent in the operating room. Additionally, frozen section diagnoses are almost always performed on a few "representative" portions of tissue, resulting in potential discrepancies between the frozen section assessment and the definitive margin status which becomes only available once the entire tissue has been processed post-operatively. Therefore, there is a significant technological and clinical need for methods capable of rapid and reliable evaluation of excised tissues in real time.

Ex vivo imaging strategies have already been proposed as potential tools for surgical margin assessment. Mahadevan-Jansen and coworkers have successfully applied contact probe autofluorescence and diffuse reflectance spectroscopy, and a spectral imaging to classify positive and negative margins of excised breast specimens with high sensitivity and specificity (Keller, 2010). However, a quantitative analysis using images was not performed. Pogue and co-workers used confocal reflectance microscopy and spectrograph to raster-scan *ex vivo* tumors margins and obtained mainly quantitative scattering parameters associated with tissue morphology (Krishnaswamy, 2009). Similar to previous wide area spectroscopic imaging, the non-probe method required some correction to deal with lines shape spectra if the technique was used to acquire quantitative absorption parameters (Keller, 2010). A

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quantitative optical imaging device to assess breast tumor margins was developed by Ramanujam *et al.* (2009) using diffuse reflectance spectroscopy for the extraction of scattering and absorption information. In this work the margin surface is obtained by manually translating an imaging probe that contacts the specimen in a container through pre-drilled holes with 5 mm center to center spacing.

Spectral imaging devices for the assessment of excised tissue have two major advantages over traditional practices of surgical margin assessment: (1) real time analysis, with the benefit of reducing patient anxiety and avoid potential follow-up surgery; (2) whole area assessment, with the benefit of reducing the probability of missing a lesion. These devices can have a huge value in breast-conserving surgery or in endoscopic mucosal resection (EMR). Several studies have suggested that in breast-conserving excision of cancer re-operation for positive surgical margins discovered after the surgery may be required in up to 50% of all cases (Allweis, 2008), and that EMR achieves complete excision of dysplastic lesions in only 4% of the cases (Mino-Kenudson, 2005).

In this paper, we present a new and complementary strategy to enable real-time comprehensive assessment of surgical margins in excised tissues. We have developed an *ex vivo* spatial high-resolution quantitative spectroscopy (QS) scanning platform which enables integration of different optical modalities to provide quantitative tissue information that correlate to disease state of the surrounding tissue. Wide area imaging of excised tissue is achieved by mechanically scanning an optical probe, with variable spatial resolution. Tissue samples are placed for analysis in a flat platform, which enables an equal pressure across time and an equal distance between probe and tissue throughout the analysis.

The developed scanning platform currently integrates diffuse reflectance spectroscopy (DRS) and intrinsic fluorescence spectroscopy (IFS) for the extraction of several spectroscopic parameters, but it is adaptable to assemble many other optical modalities. DRS and IFS have been first implemented on the scanning platform since they have shown great ability for the detection of neoplastic diseases by assessing different spectral features associated with normal and cancerous tissues (Georgakoudi, 2001); (Tunnell, 2003); (Yu, 2008). These modalities provide quantitative information about biochemical and structural tissue attributes, from which diagnostic algorithms can be developed.

Diffuse reflectance spectra from tissues are used to extract information about hemoglobin concentration and saturation, and light scattering parameters using a well-developed model based on the diffusion approximation of light propagation in tissue. DRS provides information about the morphology and biochemistry of the bulk tissue (Zonios, 1999). Intrinsic fluorescence is the fluorescence unaffected by tissue scattering and absorption, and is obtained using the diffusely reflected light to remove spectral distortions. The relative contributions of the endogenous tissue fluorophores (e.g., NADH and collagen) can be extracted from the intrinsic fluorescence (Müller, 2001). Several studies of reflectance and fluorescence for tissue diagnosis using optical fiber contact probes for light delivery and collection have been performed in different anatomic sites (Bard, 2006); (Chang, 2005); (Georgakoudi, 2001); (Müller, 2003). Despite their potential, contact probe techniques commonly suffer from undersampling. The proposed *ex vivo* QS scanning platform overcomes this drawback since it extends spectral diagnosis to the imaging mode, enabling wide area surveillance of tissue *ex vivo*.

This paper describes the design and feasibility studies of a multi-modal scanning platform for an intra-operative medical device that is able to perform a rapid, real-time, detailed, and reliable quantitative spectroscopic analysis of tissue surfaces. The major benefit of this "adaptable scanning platform" concept is that it is not limited to only one optical modality, enabling the selection of the appropriate technique for each margin assessment, or to use a combination of different techniques.

2 METHODS

Given that *ex vivo* tissue analysis does not have typical restrictions of *in vivo* imaging such as imaging geometry, surface contour, patient motion and the like, spectroscopic mapping of an arbitrarily wide area is achieved by mechanically scanning an optical probe in an inverted geometry. Spectroscopic data are taken one grid at a time with variable grid-to-grid (GTG) distance and field of view (FOV). In our instrument, the *ex vivo* tissue surface under examination can have a variable size from 2 square mm to 4 square cm, as well as variable resolution, which can be as high as a quarter of the spot size since both GTG distance and FOV are controllable. Still, if necessary, the area for tissue analysis can be easily increased in the future by

including a larger sample holder to the scanning platform. Using model-based diagnostic algorithms, this instrument will be able to correlate spectroscopic parameters with disease status in real time.

The instrument's contact probe, i.e. the FastEEM probe (Tunnell, 2003), consists of a single light delivery fiber surrounded by six collection fibers that collect light from tissue and deliver it back to the spectrograph (all seven fibers with 200 μm core and NA = 0.22). All fibers are fused together at the tip and polished at 17 degree angle to provide the overlapping of detector and collector optical cones. For spectroscopic scanning, we use only one of the collection fibers to collect tissue reflectance and fluorescence from a spot size of approximately 500 μm. The probe parameters are incorporated in our reflectance (Zonios, 1999) and fluorescence (Müller, 2001) models. Wide area coverage is achieved by scanning the light spot over the tissue using XY mechanical scanning.

Figure 1 depicts the schematic diagram of the instrument. To perform DRS measurements, white light from a 75W CW xenon arc lamp (Oriel Instruments, USA) is coupled via the delivery fiber, to illuminate a "diagnostic spot" of ~0.5 mm in diameter on the tissue sample. DRS signal from the sample is collected, with adjacent collection fibers, and coupled to a spectrometer (USB 2000+, Ocean Optics, USA). A personal computer equipped with Labview 8.5.0 software and DAQ data acquisition board NI PCI-6221 (National Instruments, USA) are used to control and coordinate the various components, including the GTG distance and FOV of the 2-D mechanical scanning (M-605.1DD and M-126.DG1, Physik Instrument, Germany). Spectroscopic data from the spectrometer and 2D stage positions are acquired and analyzed. Same resources, data handling and data acquisition are utilized for IFS measurements, except the light source is a pulsed diode pumped solid state laser that delivers 355 nm light pulses of duration ~0.6 ns and energy ~0.26 μJ at ~38 kHz (SNV-40F-000, Teem Photonics, France). Note that different measurements are accomplished through switching the excitation sources with an installed flipping mirror. Without any significant changes to the scanning engine, the integration of other optical modalities, such as hyperspectral, infrared or Raman, would only require add-on and data acquisition to the platform. This all-in-one device could be a powerfull tool in clinical tissue diagnostic. For the measurements, liquid phantoms and tissue samples were placed in removable ultraviolet glass Petri dishes, mounted on a custom sample holder.

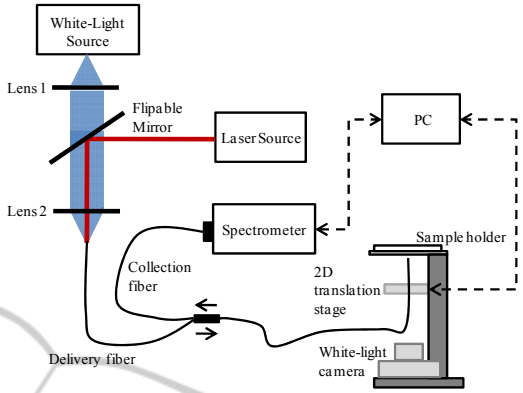

Figure 1: Schematic layout of the *ex vivo* QS scanning platform.

3 RESULTS

3.1 System Calibration

Calibration was performed using liquid phantoms with known scattering, absorption and fluorescence properties. These phantoms were constructed by a mixture of water based intralipid - scatterer - (Fresenius Kabi AG), hemoglobin - absorber - (Sigma Aldrich Co.) and furan - fluorophore - (Lambda Physik) at various concentrations. This fluorescent dye was selected because it has an excitation and emission spectra similar to that of collagen, which is an endogenous tissue fluorophore important for diagnosis. All the measurements were performed using a wavelength range from 350 to 700 nm for DRS and 380 to 700 nm for IFS.

The accuracy and capability of the system for reflectance measurements was then carried on using several combinations of various concentrations of intralipid, furan (0.5 μg/mL) and hemoglobin (0.6, 1, and 1.5 mg/mL). Reflectance spectra were acquired from one spot in each sample. All the spectra were normalized by a reflectance standard (Labsphere SRS-20) in order to remove spectral distortions and spatial inhomogeneities related with the instrument's spectral and spatial responses.

Figure 2 shows the calibrated DRS spectra, from the same position on the sample, for different phantoms. We use DRS for the extraction of diagnostic information: by fitting the reflectance spectrum to the diffuse scattering model described by Zonios et al. (1999) three DRS parameters were extracted for each pixel: A, the reduced scattering

coefficient at the reference wavelength; *B*, related to the average scatter size; and *cHb*, the total concentration of hemoglobin. However, it is important to notice that in the presented results all the samples were exposed to air and, thus, their estimated oxygen saturation is close to 1.0.

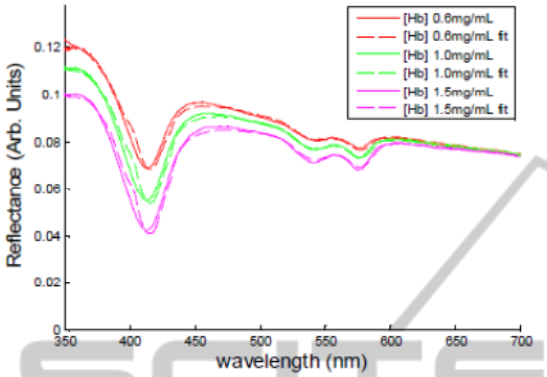

Figure 2: Calibrated reflectance spectra (solid lines) measured on different tissue phantoms. The best fit spectra are also plotted (dashed lines). The characteristic absorption bands of hemoglobin at 420nm, 540nm, and 580nm are clearly visible.

Optimal fits were obtained between the measured and computed spectra from the samples. From the excellence agreement, the computed spectra give the correct values of reflectance spectroscopy parameters, which ensure that the instrument can accurately measure the scatter and absorber parameters. The values for parameters *A*, *B*, and *cHb* are compiled in Table 1.

Table 1: Reflectance parameters (*A*, in mm^{-1}, *B*, and *cHb* in mg/mL) measured from tissue phantoms with different hemoglobin concentrations.

Parameters	[Hb] = 0.6	[Hb] = 1.0	[Hb] = 1.5
A	1.074	1.074	1.070
B	0.289	0.278	0.230
cHb	0.619	1.002	1.472

The accuracy of fluorescence measurements was assessed using the same set of phantoms. The fluorescence at each spot is analyzed using IFS: reflectance measurements are used to correct the bulk fluorescence spectra (affected by scattering and absorption) using the model described by Müller *et al.* (2001) to extract the IFS spectra. Figure 3 shows the fluorescence spectrum of pure furan in water (blue line), and the several bulk and IFS spectra measured using phantoms with different hemoglobin concentrations.

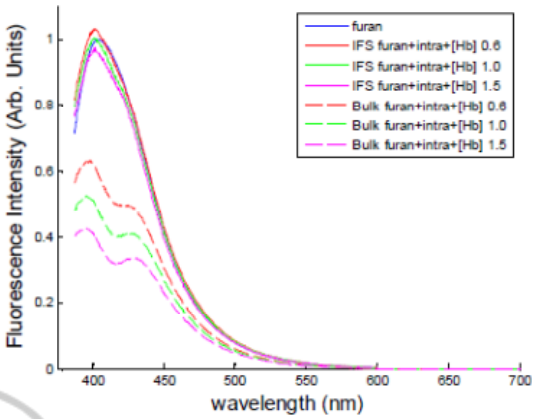

Figure 3: Bulk fluorescence spectra (dashed lines) measured on different tissue phantoms. The corresponding intrinsic fluorescence spectra (IFS) are also plotted (solid lines). The blue spectrum is the fluorescence measured from pure furan in water. Note that the calculated IFS spectra, which are independent to the absorbers and scatterers and the raw spectrum of pure furan are well overlapped.

As expected, the data confirms that bulk fluorescence spectra vary considerably with hemoglobin concentration in opposition to the IFS spectra. The excellent agreement between the IFS spectra and the spectrum of pure furan in water indicates that IFS method can be used to remove the distortions caused by tissue scattering and absorption. These data provide evidence for accurate calibration of our QS scanning platform.

3.2 Brain Tissue Imaging

The performance characteristics of the *ex vivo* QS system were demonstrated using an inherently high contrast sample with sharp regional boundaries (a section of formalin-fixed human brain cortex with gray and white matter, with an approximate size of 2 cm by 2 cm). A diffuse reflectance map of the brain is shown in Figure 4(a), obtained using a step size of 125 µm, and an integration time of 3 ms at each point. In this image, fine detail and high contrast between the gray and white matter of the brain cortex is clearly visible. For demonstration, high-resolution spectral maps of the scattering parameter, *A*, and measured hemoglobin concentration are shown in Figure 4(b) and Figure 4(c), respectively. As expected, it is revealed a higher hemoglobin concentration on gray matter (related with higher blood volume) (Hamberg, 1996). These results demonstrate the ability of *ex vivo* QS scanner to provide spectral contrast based on tissue parameters.

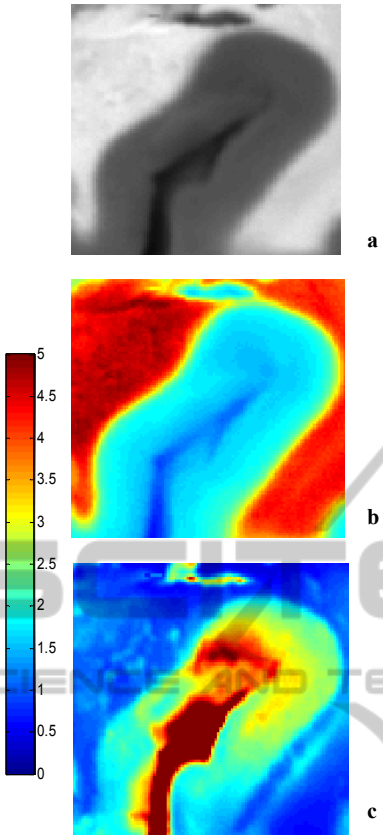

Figure 4: Representative QS images of brain cortex taken with the proposed spatial high-resolution scanner: a) total reflectance maps (in arbitrary units); b) quantitative map of the scattering parameter A (mm^{-1}); c) quantitative map of hemoglobin concentration (mg/dl). Each box represents approximately a 1 x 1 cm scanning area.

The concept has thus been demonstrated with the developed bench-top platform using DRS and a small biological sample. However, as previously mentioned, the system is not limited to DRS and IFS. Other modalities, such as Raman and infrared spectroscopy can be readily integrated on our platform to provide additional tissue information.

4 CONCLUSIONS

A quantitative multi-modal spectroscopy scanning platform was constructed for assessing *ex vivo* tissue biochemical and morphological information. This newly developed instrument is ideal for characterization of surgically excised tissue margins and provides two major benefits over the current practice: (1) reduce patient anxiety and avoid follow-up surgery because on-the-fly real time data analysis can be performed; (2) reduce the probability of missing a lesion because the whole *ex vivo* tissue area can be assessed. The proof of principle has been demonstrated in this study with bench-top prototype for quantitative spectroscopic scanning of a biological sample while the construction of the compact clinical unit is in progress. For the clinical system several instrumentation and software advances are needed: reduction of system size, increase in collection and analysis speed, and improved user interface with diagnostic algorithms.

Further investigation is also needed to address the effect of excision in quantitative hemoglobin and fluorophores measurements. For instance, excised specimens contaminated by the presence of surface blood may absorb the majority of reflected light, significantly reducing the reflectance signal (Volynskaya, 2008). In addition, some fluorophores (e.g. collagen) might be stable in excised tissue, whereas others (e.g. NADH) might degrade over time, precluding an accurate extraction of its concentration.

The extraction of quantitative optical parameters, such as hemoglobin and collagen, has proven to be helpful for the differentiation of normal and malignant tissues. A recent study from Volynskaya et al. (2008) has successfully demonstrated that higher hemoglobin concentrations and higher collagen values were more likely to be found in ductal carcinoma of breast tissue than in normal breast tissue, and thus could be used as diagnostic parameters.

The *ex vivo* spectroscopic scanning platform concept is not restricted to only DRS and IFS and should be extended in the future to other optical modalities in order to gather additional and complementary diagnostic information.

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