Human Bladder Cancer Diagnosis Using Multiphoton Microscopy

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Abstract

At the time of diagnosis, approximately 75% of bladder cancers are non-muscle invasive. Appropriate diagnosis and surgical resection at this stage improves prognosis dramatically. However, many of these lesions are small and sometimes flat, and are apt to be missed by conventional white-light cystoscopes. Furthermore, it is difficult to assess the surgical margin for negativity using conventional cystoscopes. Thus, Multiphoton endoscopes with high magnification and resolution are currently under construction at Cornell University, Ithaca, under the leadership of Professor Watt Webb. The current study is designed to confirm that Multiphoton imaging (using a custom assembled bench-top Multiphoton microscope) can indeed identify cancers in fresh, unfixed human bladders. Fresh bladder biopsy specimens from the operating room are brought directly to the Multiphoton microscope for imaging. Immediately after Multiphoton imaging (lasting one to one and a half hours), the specimens are taken to the Pathology department for routine histopathological workup. Multiphoton images are acquired in two channels: (1) broadband autofluorescence from cells, and (2) second harmonic generation, mostly by tissue collagen. Multiphoton images are compared with current gold standard of histopathology, hematoxylin/eosin (H&E) stained thin sections from the same specimen. Based on a “training set” and a very small “blinded set” of samples, we have found excellent correlation between the Multiphoton and histopathological diagnoses. A larger blinded analysis by two independent uropathologists is currently in progress. We expect that the conclusion of this phase will provide us with diagnostic accuracy estimates, as well as the degree of inter-observer heterogeneity.

Clinical Background

In the United States, bladder cancer is the fourth and the eighth most common malignancy among men and women, respectively; with over 50,000 new cases and ~12,000 deaths annually1. At the time of diagnosis, approximately 75% of bladder cancers are locally contained, i.e., they are non-muscle invasive2. However, many of these lesions are small and flat, the most aggressive of them being the Carcinoma in situ (CIS), whose presence has been identified as an important prognostic factor for the recurrence and/or progression of bladder cancer3. A recent estimate suggests that conventional white light cystoscopy (trans-urethral bladder endoscopy and biopsy) is apt to miss up to 42% of the CIS4. Thus the surgeon is often faced with a dilemma in the case of suspicious “equivocal” cases: too few biopsies will increase false
negative rate, and too many will increase cost and patient morbidity. As it is, cystoscopy requires anesthesia and overnight hospital stay followed by a healing period before full treatment resumption. This makes bladder cancer the fifth most expensive cancer in terms of total medical care expenditures, primarily because of the long term survival of the patients, and the need for lifelong monitoring (most often by biopsies) and treatment.

We propose that multiphoton microscopy (MPM) \textsuperscript{6-8}, especially when adapted to a multiphoton endoscope (MPE) format, will be a very powerful diagnostic tool for early bladder cancer. It has the potential to better identify early lesions, especially CIS, during the first cystoscopy, and the ability to image the resection margin to confirm negativity.

**Multiphoton Microscopy**

Briefly, MPM involves the illumination of a sample by near-infrared light from a femtosecond pulsed laser, which is used to excite fluorescence from the naturally occurring fluorophores residing at the focal volume. Intrinsic fluorophores abundantly present in most cells include reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotides (FAD). In addition to cellular autofluorescence, MPM allows the identification of non-centrosymmetric structures such as collagen by utilizing a higher order scattering phenomenon called Second Harmonic Generation (SHG).

Using a combination of autofluorescence from cells and SHG signal from the connective tissue rich in collagen, MPM imaging can provide detailed information on tissue architecture and cellular morphology, comparable to those obtained from standard H&E stained histopathology slides. All this can be done with minimal phototoxicity to cells, and images of adequate quality can be obtained up to depths of ~500 microns in a few seconds.

**Suitability of the human bladder as a first prototype organ for MPM/MPE**

The human bladder is an ideal candidate for the standardization of MPM imaging and MPE methodology, since it has a simple architecture \textsuperscript{9}, whose perturbation by cancer can be easily detected at cellular and tissue organization levels. The bladder lumen is lined by the ~7-cell layer transitional endothelium or urothelium. The innermost layer of the urothelium surrounding the lumen is composed of the so-called umbrella cells, with a unique morphology that can be identified by MPM. The rest of the endothelial layers are highly organized in columns, and any perturbation of this linear arrangement will also be visible by MPM. The endothelial layer is followed by lamina propria, composed of connective tissue (containing collagen) and scattered smooth muscle cells, which in turn is followed by muscularis propria, and finally, by a layer of perivesical soft tissue, composed of fat, fibrous tissue and blood vessels.

Most bladder cancers originate in the endothelial layer and the early cancers we are interested in may show an increase in the thickness of the urothelial layer (hyperplasia), and/or a penetration of the urothelial cells into the lamina propria (breaching the clear separation of the cellular layer of the
endothelium from the mostly collagenous lamina propria)10.

Materials and Methods

Fresh bladder biopsy specimens {either trans-urethral resection of bladder (TURB) or cold cup biopsies} are placed in normal saline immediately after excision, and the specimen bottles, placed in ice, are brought directly to the Multiphoton microscope for imaging. In the imaging facility, the biopsy specimen is placed on a small tissue culture dish with a central well, with the urothelium oriented on the top. If necessary, the specimen is stabilized in optimum orientation using nontoxic modeling clay, and a coverslip (#1 glass) is placed on top of the urothelium (in order to avoid sticking of the biopsy piece to the water immersion objective due to capillary action).

The specimen, thus oriented, is imaged using a custom-built Multiphoton imaging system consisting of an Olympus BX61 upright microscope and a BioRad 1024 scanhead. The specimens are excited using a tunable Ti-Sapphire laser (Mai Tai, Spectra-Physics), tuned to 780 nm. The laser power under the objective is controlled through a Pockel Cell (Conoptics). Images are collected in two channels: (1) SHG signal centered around 390 nm (±35 nm), and (2) broadband autofluorescence at 420-530 nm.

Images are acquired at two magnifications: (1) Low magnification for obtaining overall architectural information (4X, 0.28 NA non-immersion objective). This allows us to image 3 mm² frames at 6 μm/pixel resolution. (2) High magnification for obtaining detailed cellular and local architectural information (20X, 0.95 NA water-immersion objective). When not using any digital zoom, this allows us to image 614 μm² frames at 1.2 μm/pixel resolution. Higher digital zooms are used to increase magnification, if necessary. When imaging is done using the 20X objective, a drop of normal saline was placed on the coverslip to achieve water immersion. All images are obtained as stacks of optical sections through the entire depth of the specimen from where reasonable signal can be obtained. Typically, we are able to image 400-450 μm into the tissue with the 4X objective, and 200-250 μm with the 20X objective.

For all images presented here, the SHG signal has been color-coded red, and the autofluorescence signal has been coded green. Metamorph (Molecular Dynamics, Inc.) and Adobe Photoshop were used for image color-coding, minimal processing (adjustment of brightness and contrast, placement of scale bars and cropping) and presentation.

Immediately after imaging, the biopsies are dropped in standard formalin, and taken to the Pathology department for routine surgical pathology workup and diagnosis. This includes gross morphological assessment of the tissue, as well as microscopic evaluation of H&E stained slides of thin sections prepared from the tissue.

In the initial “training phase”, the uropathologist, the Multiphoton microscopist, and the urologists got together, and compared the Multiphoton images and the H&E slides from the specimen, to learn to identify comparable features. We also used the official surgical Pathology report on the case as
a reference.

Very recently, we have begun a blinded trial to assess the diagnostic accuracy of Multiphoton images.

Here, the uropathologists are given de-identified Multiphoton images alone (a set of images representing all the images acquired from a given specimen), and are expected to make a diagnosis based on this information alone. They are expected to classify the specimen into one of the following classes: (1) Normal urothelium; (2) Papillary lesion: (a) Benign, (b) Ta, low grade, (c) Ta, high grade; (3) Carcinoma in situ; (4) Invasive cancer; (5) Other.

**Results**

Initial side-by-side comparisons of the Multiphoton images and H&E stained histopathology images from the same specimen showed a high degree of correlation of diagnostic features, as described below.

**Benign papillary lesion**

We were able to visualize the urothelium (autofluorescence from cells color-coded green) as separate from the underlying lamina propria (a region rich in collagen, and hence showing SHG signal color-coded red). Please note the clear boundary between the urothelium and the lamina propria, suggesting the absence of invasive tumor.

![Image A](image1.png) ![Image B](image2.png)

**Low grade papillary tumor**

Once again, we can visualize the urothelium as separate from the underlying lamina propria. The lamina propria forms “stalks” that extend into
proliferating urothelium. A profusion of papillary structures are seen in cross section. Please note that the cells look relatively normal and homogeneneous, which indicates that it is a low grade tumor.

High grade papillary tumor

Here, at low magnification, the cells look highly compacted and arranged in “whorls” (profusion of cells with scant lamina propria around cell clusters). At high magnification, we observe extensive cell heterogeneity (panels C and D), as well as cells with very large nuclei (panels E and F).
Discussion

What is wrong with the current standard of care for bladder cancer patients?

As discussed in the introduction, bladder cancer predominantly presents as non-muscle invasive cancer, which, if detected and completely resected, would considerably improve prognosis. However, the current diagnostic techniques are fraught with limitations. Current standard of practice includes two diagnostic tests to rule out urothelial carcinoma in patients presenting with microscopic or gross hematuria, which are the presenting symptoms for over 80% of the patients. These tests are: (1) urine cytology, and (2) white light cystoscopy. Since the tumor is in direct contact with the urine, tumor cells are frequently shed into urine, and hence a urinary analysis for the presence of malignant cells seems a prudent approach. However, although this technique has high specificity (94% median), the median sensitivity is only 35%. Conventional white light cystoscopy, while quite efficient at detecting relatively large papillary tumors, performs poorly in identifying flat lesions (particularly CIS), dysplasia, multifocal growth and microscopic papillary tumors.

If tumor is detected by the above methods, the patient is then subjected to TURB (trans-urethral resection of bladder) biopsy procedure. This involves white light cystoscopy, along with biopsy removal from suspicious regions, which are then assessed by H&E histopathology to determine stage (degree of invasiveness) and grade (degree of transformation of the cells). Whereas surveillance cystoscopy is routinely done as an outpatient procedure, TURB biopsy procedures require general anesthesia, overnight hospital stay, and substantial morbidity. If the biopsies reveal only localized (non-muscle invasive tumor), then the patient may or may not be treated pharmacologically (depending on exact stage and grade), but is routinely monitored for recurrence, most often via repeat cystoscopies.

One of the biggest problems with the clinical management of non-muscle invasive bladder cancer is the very high recurrence rate (31-78% at 5 years). This high recurrence rate, along with relatively high long term survival, makes bladder cancer the most expensive cancer at the patient level, with ~60% of the costs attributable to treatment of recurrences. Interestingly, a study investigating the source of “recurrences” of tumor on repeat cystoscopy found that up to ~50% of the apparent recurrences were in fact pre-existing tumors that were missed during the first cystoscopy.

If the patient is found to have muscle invasive bladder tumor upon TURB biopsy, the standard of care is radical cystectomy.

What can be done to rectify some of the problems with the standard of care?

As experience accumulates from other surgical specialties where “multimodal imaging” has become more common (such as colorectal cancer, or Barrett’s
Esophagus), it is becoming clear that at least two types of endoscopic techniques need to be used in conjunction. In general, these can be classified into “primary detection” techniques and “targeted imaging” techniques\textsuperscript{15}. The primary detection would include localizing tumor in an organ. A good primary detection technique will be able to detect tumors with high specificity and sensitivity. White light endoscopy is the conventional modality, and is being currently supplemented with several newer “red flag” techniques, which are designed to draw the attention of the endoscopist to regions that might contain early tumors or dysplasia. Such techniques include chromoendoscopy (using contrast agents such as methylene blue or indigo carmine) and narrow band imaging (NBI). NBI involves the illumination of the mucosal surface with narrow bands of blue and green light, which, due to their short wavelength, penetrate only the superficial layers. Furthermore, the blue light is preferentially absorbed by the hemoglobin in the blood vessels, which makes them appear dark. Together, they produce increased contrast, allowing better visualization of mucosal and vascular patterns\textsuperscript{16}.

One such technique, that has been shown to be especially useful in improving detection of early bladder cancer, is the so-called “fluorescence cystoscopy”\textsuperscript{17}. The most commonly used agent is 5-aminolevulinic acid (ALA). ALA is a normal component of the heme biosynthetic pathway, and one of the intermediates in this pathway is Protoporphyrin IX, which, when excited by blue light, gives a bright red fluorescence. When treated with ALA, tumor cells preferentially accumulate Protoporphyrin IX, and thus appear brighter when illuminated by blue light. The mechanism of this preferential accumulation is not clear, and is possibly a result of multiple causes. One interesting observation is that many tumor cells have a reduced activity of the enzyme ferrochelatase, which normally converts Protoporphyrin IX to heme, possibly due to a diminished supply of iron. For detection of early bladder cancers during cystoscopy, ALA is instilled intravesically (by filling the bladder with a ALA containing buffer for several hours), thereby minimizing many side effects of ALA such as photosensitivity.

Diagnosis of early bladder cancer, and especially CIS, is dramatically improved with ALA treatment followed by blue light illumination. Several recent clinical trails comparing ALA-mediated diagnosis with conventional white light cystoscopic diagnosis found a mean increase in sensitivity of ~20%, with sensitivities for ALA-mediated diagnosis being >95\%\textsuperscript{11}. Indeed, an outcomes study looking at an 8-year recurrence free survival showed 71% positive response in the ALA-mediated diagnosis group, compared to only 45% in the conventional white light cystoscopy group.

However, the specificity of this method is relatively low and quite variable\textsuperscript{17-19}. This high false positive rate appears to be associated with changes in Protoporphyrin IX accumulation in various types of non-neoplastic cells, chief among them being sites of inflammation\textsuperscript{18}. This also results in much higher false positive rates in patients with a recent history of cystoscopic or pharmacological treatment for bladder cancer\textsuperscript{20}.

Newer fluorescence agents based on the ALA model are currently being tested\textsuperscript{21}. One of these is the hexyl ester of ALA (HAL), which, being more hydrophobic, is taken up by cells more readily, thus requiring lower
concentrations and shorter instillation periods. Hypericin, extracted from Hypericum perforatum (St. John’s wort) is being investigated as another potential alternative. Early studies indicate that this agent may label tumor cells with high sensitivity as well as specificity.

“Targeted imaging” techniques, on the other hand, allow a more detailed evaluation of the sites picked up as “of potential interest” by the red flag techniques described above. These techniques have often been referred to in the literature as “optical biopsies”. Primary among these techniques is confocal microendoscopy, which can provide subcellular details of the superficial mucosal layers. Confocal microendoscopy is typically conducted in the fluorescence mode, although confocal reflectance endoscopy is also being investigated as a potential option. Other optical biopsy techniques currently under investigation include optical coherence tomography and Raman spectroscopy.

**Multiphoton endoscopy: a new frontier in optical biopsy?**

Confocal fluorescence endoscopy, although it can produce images of resolution comparable to gold standard histology, has several limitations, the most important ones being the necessity of using exogenous contrast agent such as Fluorescein for optimal imaging. Also, the relatively short wavelength of excitation significantly limits light penetration into the tissue, allowing the imaging of only the superficial layers of cells. There are also concerns about photodamage, since although signal is collected from only one focal plane at a time, the illuminating light can bleach and potentially photodamage cells in the entire illumination cone. Optical coherence tomography, while eliminating some of these concerns, has a much lower resolution than confocal imaging.

Our preliminary results presented here strongly suggest that images of adequate resolution and contrast can be obtained from fresh, unstained human tissue using MPM (using a combination of autofluorescence and SHG signals). When compared with H&E stained histopathology slides prepared from the same specimen, we obtain good correlation of the diagnostic features between the MPM and the H&E images. Of course, the ongoing blinded studies will tell us whether the current imaging approach (two signals: SHG and broad band fluorescence) will give us enough diagnostic accuracy to make reliable diagnoses in real time in the eventual endoscopic format.

While thinking of extending the MPM imaging technology from ex vivo biopsies to endoscopic imaging in real patients, several factors need to be considered. One concern that is common to all optical biopsy techniques is that these techniques, because of the limited field of view and relatively slower image acquisition speeds, will necessarily require a combination with another high specificity primary detection technique. It is possible to visualize that ALA-mediated imaging might be an optimal system to consider, primarily because it has been demonstrated to detect early tumors in bladder with high sensitivity. The lower specificity of ALA detection may be rectified during the cystoscopic session by switching to MPM imaging mode in the suspicious regions. However, ALA treatment does require instillation of an exogenous reagent, thus adding to total treatment time and potential
complications (e.g., allergic reactions to ALA have been reported). Also, to the best of our knowledge, no thorough investigation has been carried out to test whether the presence of ALA or Protoporphyrin IX in cells will affect their signatures during MPM. If these limitation present as significant challenges in clinical adaptation, other alternatives to improve contrast at low magnification, such as NBI or confocal reflectance may be investigated as better options for pairing with Multiphoton endoscopy.

Indeed, this is a common trend in other cancer diagnosis scenarios as well. Attempts are currently underway to further increase the range of techniques at the disposal of an endoscopist. An example is a recent international, multi-center feasibility study that involved tri-modal imaging options (high resolution video endoscopy, autofluorescence imaging and NBI) within a single endoscopic device, for diagnosis of early neoplasia in Barrett’s esophagus. The results indicated that combined high resolution endoscopy and autofluorescence imaging had very high sensitivity, but poor specificity. The false positive rate of this combination was reduced by a detailed inspection of the Barrett’s mucosa with NBI.

Another issue that will need further investigation is the maximum tolerated light dosage – both to avoid frank tissue burning, as well as ensuring that no insidious negative effects, such as increased mutation rates, were caused as a result of illumination with a femtosecond pulsed laser source, that would be necessary for this type of imaging.

However, assuming that Multiphoton endoscopy, along with a suitable primary detection method, can be translated to clinical use, it can have tremendous impact on current practice, since it will allow (1) resection of only what is truly malignant (high specificity), (2) identification and resection of early lesions, and (3) assessment of resection margins to ensure negativity. Also, since routine surveillance cystoscopy (without the necessity to remove biopsies) can be done as an outpatient procedure, it is likely to reduce the overall cost of management of bladder cancer survivors. TURBs will then only be scheduled in follow-up cases when surveillance Multiphoton endoscopy confirms the presence of new tumors.

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