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Real-Time Near-Infrared Fluorescence-Guided Identification of the Ureters using Methylene Blue

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Abstract

Background—The aim of this study was to determine whether the invisible near-infrared (NIR) fluorescence properties of methylene blue (MB), a dye already FDA-approved for other indications, could be exploited for real-time, intraoperative identification of the ureters.

Methods—The optical properties of MB were quantified *in vitro*. Open surgery and laparoscopic NIR fluorescence imaging systems were employed. Yorkshire pigs were injected intravenously with: 0.1 mg/kg MB (n = 8), 10 mg furosemide followed by 0.1 mg/kg MB (n = 6), or 0.5 mg/kg MB (n = 6). The contrast-to-background ratio (CBR) of the kidney and ureters, and MB concentration in urine, were quantified.

Results—Peak MB absorbance, emission, and intensity in urine occurred at 668 nm, 688 nm, and 20 μ M, respectively. After intravenous injection, doses as low as 0.1 mg/kg MB provided prolonged imaging of the ureters, and a dose of 0.5 mg/kg provided statistically significant improvement of CBR. Pre-injection of furosemide increased urine volume but did not improve CBR. Laparoscopic identification of the ureter using MB NIR fluorescence was demonstrated.

Conclusions—Ureteral imaging using MB NIR fluorescence provides sensitive, real-time, intraoperative identification of the ureters during open and laparoscopic surgeries.

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INTRODUCTION

Although ureteral injuries are uncommon, with a reported incidence rate between 0.007% and 1.8%,^{1–3} recognition of such injury is often delayed, with the average time to detection ranging from 5.3 to 180 days.^{4,5} Importantly, intraoperative detection of such injuries can reduce morbidity and medical costs.^{3,5,6} To help avoid injury, intravenous (IV) pyelography (IVP), retrograde pyelography, or urological computed tomography (Uro-CT) can be performed preoperatively, however, none of these imaging techniques provides real-time guidance during the actual procedure. The need for sensitive, real-time, intraoperative identification of the ureters has also increased along with the number of minimally-invasive procedures being performed. Assimos compared the incidence of iatrogenic ureteral injuries between the pre-laparoscope and post-laparoscope era and showed that the incidence of iatrogenic ureteral injuries was significantly higher in the latter.⁷ Thus, an ideal imaging technique for ureteral identification should be available during both open and minimally-invasive surgeries.

Even though it is invisible to the human eye, near-infrared (NIR) light between 700 nm and 900 nm has significant advantages for intraoperative imaging, including low absorption, low scatter, and low autofluorescence.⁸ We have previously demonstrated a real-time intraoperative ureteral identification technique using an NIR fluorescence imaging system combined with the IV injected fluorophore IRDyeTM800-CW carboxylic acid (CW800-CA).⁹ However, CW800-CA is a tetra-sulfonated heptamethine indocyanine that is not approved by the US Food and Drug Administration (FDA) for human use. In this study, we hypothesized that the clinically available dye methylene blue (MB) may have NIR fluorescence properties that would permit real-time, intraoperative visualization of the ureters.

MATERIALS AND METHODS

Reagents and Animals

MB (methylene blue injection USP, 1%, 10 mg/ml, 31.3 mM) was from Taylor Pharmaceuticals (Buffalo Grove, IL). Furosemide was from American Regent (Shirley, NY). Animals were studied under the supervision of an approved institutional protocol. Female Yorkshire pigs (E. M. Parsons and Sons, Hadley, MA) were 10 to 12 weeks old, and 31.8 to 40.3 kg (mean; 36.7 kg). Pigs were induced with 4.4 mg/kg intramuscular Telazol (Fort Dodge Labs, Fort Dodge, IA), intubated, and maintained with 2% isoflurane (Baxter Healthcare Corp., Deerfield, IL). Electrocardiogram, heart rate, oxygen saturation (SpO₂), body temperature and blood pressure were monitored during all experiments. Saline was given at 350 ml per hour through a central venous catheter. Urine was obtained through a 10 Fr catheter placed in the bladder.

Measurement of Optical Properties

Absorbance and fluorescence of MB in urine were measured as described in detail previously,¹⁰ except urine was diluted 2-fold in distilled water for absorbance measurements to lower background. For fluorescence quantum yield (QY) measurements of MB in urine, oxazine 725 in ethylene glycol (QY = 19%¹¹) was used as a calibration standard under conditions of matched absorbance at 655 nm. For measurement of MB concentration in urine over time, calibration solutions were prepared in swine urine and sample concentration determined after measurement of absorbance in a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). For pH sensitivity measurements, the absorbance and fluorescence spectra of MB were acquired after adjustment of a phosphate-buffered saline (PBS) pH to 4, 7, or 10 using 5 N HCL or 10 N NaCl. Leucomethylene blue was prepared by dissolution of MB in 25 μ M ascorbic acid (titrated to pH 0.1 with 5 N HCl) in deoxygenated water.

NIR Fluorescence Imaging System for Open Surgery

The FLARE™ (Fluorescence-Assisted Resection and Exploration) image-guided surgery system and its high-powered light emitting diode (LED) light source have been described in detail previously.^{12,13} Color video and NIR fluorescence images are acquired simultaneously, at rates up to 15 Hz, using custom software.

NIR Fluorescence Imaging System for Laparoscopic Surgery

Light from a custom 300W Xenon light source (Wilson Associates, Weymouth, MA), equipped with filtration to remove all NIR and infrared light, was combined with light from a 500 mW, 670 nm laser diode (Thor Labs, Newton, NJ) through a custom light mixer (Qioptiq Imaging Solutions, Fairport, NY), then through a 0.6 NA fiber optic cable to illuminate a standard rigid laparoscope (10 mm diameter, 0°; Storz, Tuttlingen, Germany). The eyepiece of the laparoscope was attached to custom optics (Qioptiq Imaging Solutions), which permitted simultaneous acquisition of color video and NIR light using a HV-D27 (Hitachi, Woodbury, NY) color CCD camera and Orca-AG (Hamamatsu, Bridgewater, NJ) NIR camera, respectively.

Ureteral Imaging and Quantitative Assessment

For open surgery experiments, a standard midline laparotomy was performed, and a unilateral transrectus incision was added to expose the entire course of the ureter. The FLARE™ system was positioned 18" above the surgical field, and images recorded at time = 0, every minute from 0 to 5 min after IV injection of MB at the specified dose, then every 5 min until 75 min post-injection. All images were acquired with a 150-msec exposure time. At each time point, the fluorescence intensity (FI) and background (BG) intensity of a region of interest (ROI) over the kidney, the upper ureter, and the lower ureter were quantified using custom software. The performance metric for MB fluorescence was the contrast-to-background ratio (CBR), defined as $CBR = (FI - BG \text{ intensity}) / BG \text{ intensity}$.

Urine pH and blood pH were measured prior to MB injection and every 15 min from 10 min to 70 min. The bladder was emptied at each sampling. Animals that continuously showed $HR \geq 150$, $SpO_2 \leq 95\%$, and blood pH varying by more than ± 0.2 were excluded. Also, animals with anuria or oliguria (urine volume < 1 ml for 15 min) were excluded. Optical measurements of urine occurred within 1 h after collection.

For laparoscopic surgery experiments, the left kidney and ureter were studied in 2 pigs. Each pig was injected with 0.1 mg/kg MB as a 5-min infusion, imaged, then re-injected with 1.0 mg/kg MB as a 5-min infusion after a 90-min washout period. Images were recorded at 0, 10, and 30 min after each MB injection.

Statistical Analysis

Results were presented as mean $\pm$ SEM. An unpaired Student's t-test was employed to determine the statistical difference between two groups with a 95% confidence interval. An analysis of variance was used to analyze three or more groups. Correlation was studied using the two-tailed Pearson test with a 95% confidence interval. A *p* value of less than 0.05 was considered significant.

RESULTS

Measurement of MB Optical Properties

MB is a small molecule that can be converted to a colorless form called leucomethylene blue (LMB). This conversion is initiated by a reducing and/or acidic environment (Figure 1A).

When used at the typical clinical concentration of 1%, i.e., 31.3 mM, MB is brightly blue, but has virtually no NIR fluorescence in either PBS, pH 7.4 or swine urine (Figure 1B) due to quenching. When diluted to 20 μM (quenching threshold) or below, however, MB displays moderately high NIR fluorescence (Figure 1B), with peak excitation at 668 nm, extinction coefficient of $69,100 \text{ M}^{-1}\text{cm}^{-1}$, peak emission at 688 nm, and a QY of 4.4%, all in swine urine (data not shown). For comparison, peak excitation, extinction coefficient, peak emission, QY, and quenching threshold of MB in PBS, pH 7.4 were 665 nm, $73,000 \text{ M}^{-1}\text{cm}^{-1}$, 684 nm, 5.3%, and 12.5 μM respectively (data not shown and Figure 1B). Methylene blue absorbance and fluorescence exhibited minimal changes over a wide pH range from 4 to 10 (data not shown). In fact, ascorbic acid had to be added to a concentration of 25 μM and pH reduced to 0.1 before LMB formation was observed (Figure 1A).

Intraoperative NIR Fluorescence Imaging Systems

Optical light paths and filtration for the FLARE™ open surgery imaging system are shown in Figure 2A. Key features of the system are the ability to acquire color video (i.e., 400–650 nm “white” light) images simultaneously with NIR fluorescence images, high frame rates (up to 15 Hz), a large field-of-view of 15 cm, a long working distance (18”), and the use of safe LED light. Fluence rates used for this study were 2.5 mW/cm^2 for white light and 0.6 mW/cm^2 for 670 nm excitation light. The laparoscopic imaging system had the same basic design and performance (Figure 2B), although laser light was required for fluorescence excitation due to the relatively low NIR transmission of the fiber optics.

Real-Time Ureteral Identification during Open Surgery

Animals were divided into 3 groups. The first group ($n = 8$) was injected with a slow infusion, over 5 min, of 0.1 mg/kg MB diluted in 30 ml saline. The second group ($n = 6$) was pre-treated with 10 mg IV furosemide 1 h prior to the MB injection described for the first group. The third group ($n = 6$) was injected as described for the first group, except the MB dose was 0.5 mg/kg. The physiological characteristics of the 3 groups were statistically matched (Table 1).

In all animals, the ureter was successfully identified during the time period 10 min to 75 min post-MB injection (Figure 3). The mean CBR of the kidney, the upper ureter, and the lower ureter was significantly higher in the 0.5 mg/kg MB group (kidney: 1.96 ± 0.35 , upper ureter: 6.05 ± 0.60 , lower ureter: 6.91 ± 0.81) than in 0.1 mg/kg MB group (kidney: 1.01 ± 0.14 , upper ureter: 3.66 ± 0.47 , lower ureter: 4.32 ± 0.60), with $p = 0.016$, 0.003, and 0.015, respectively (Figure 4A). For the kidney, the upper ureter and the lower ureter, there was no statistical difference in mean CBR between 0.1 mg/kg MB without (see above), and with furosemide pre-treatment (0.88 ± 0.12 , 3.72 ± 0.43 , 4.69 ± 0.62), for which p values were 0.508, 0.921, and 0.674, respectively (Figure 4B). However, as expected, furosemide caused a vigorous diuresis (data not shown). The MB concentration in urine was comparable for 0.1 mg/kg MB doses, with and without furosemide pre-treatment, although it was significantly higher ($p = 0.014$ to 0.044) for the 0.5 mg/kg dose (Figure 4C). Neither blood pH, nor urine pH, in any of the three groups, showed any correlation with mean CBR of the upper and the lower ureters (data not shown).

Real-Time Ureteral Identification during Laparoscopic Surgery

The laparoscopic imaging system described in Figure 2B was used. Because of light losses inherent to such prototype systems, IV injection of 0.1 mg/kg MB failed to produce an acceptable CBR of the ureters. However, after IV injection of 1 mg/kg MB, the ureters could be readily identified in real-time, from a period of 10 min to 30 min post-injection (Figure 5).

DISCUSSION

In this study, we demonstrate that when adequately diluted to micromolar concentrations, MB becomes an excellent NIR fluorophore, which is unaffected by physiological changes in pH. Since a major route of excretion of MB is through the kidneys,^{14,15} urine becomes intensely NIR fluorescent after a single IV dose, which permits real-time identification of the ureters.

We hypothesized that by giving a diuretic, and thus increasing the flux of urine through the ureters, NIR fluorescence of the ureters would be increased. Even though urine flux did increase, there was neither a qualitative nor quantitative improvement in MB NIR fluorescence, suggesting that dilution of MB in urine was counteracting the expected increase in the number of moles eliminated per unit of time. It is also possible that furosemide affected MB to LMB inter-conversions,¹⁶ which would also act to limit NIR fluorescence. Additionally, in the absence of diuretic, there was little difference in MB performance when IV injection was given as a rapid bolus or a slow infusion over 5 min (data not shown). This observation is similar to results reported for using MB as a blue dye for parathyroid identification.¹⁷

The signal intensity of NIR fluorescence in the ureters is a function of MB dose, NIR fluorescence excitation fluence rate, and sensitivity of the emission optics. As shown in this study, increasing urinary concentration beyond approximately 20 μM is counter-productive, since NIR fluorescence is quickly lost due to quenching (Figure 1B). Also, since adverse reactions to MB are more common when the IV dose is $\geq 5 \text{ mg/kg}$,^{18–20} the lowest possible dose should be used. Excitation fluence rate can certainly be increased to improve the CBR, to reduce the camera exposure time, and to reduce MB dose; however, the 656 to 678 nm excitation light can imbue the surgical field with a reddish tint, which places a limit on how high a fluence rate will be tolerated. Finally, care in the emission optics is of critical importance, as evidenced by our NIR fluorescence laparoscope prototype. Because of the light losses inherent to using an off-the-shelf laparoscope, higher MB dosing and longer camera exposure times were required to obtain CBR similar to those of the open surgery system.

Recently, a new radioactive technique for intraoperative ureteral localization was described. Tc-99m-labeled diethylenetriamine pentaacetic acid (^{99m}Tc-DTPA) was administered IV as a contrast agent and resulted in excellent signal-to-background ratio, as high as 465% on average.²¹ However, ^{99m}Tc-DTPA requires special handling, exposes patient and caregivers to ionizing radiation, does not provide imaging of the surgical field, and is expensive. On the other hand, NIR fluorescence provides real-time (up to 15 images per second) imaging of a large field of view, our custom imaging system provides surgical landmarks via the color video image, and the contrast agent (MB) is non-radioactive and available at every institution. We also observed prolonged visualization of the ureters for up to 75 min after a single MB injection. For longer procedures, MB can be re-injected as many times as needed, with each injection providing an additional 60 to 75 min of visualization (data not shown). Because of these features, ureteral dissection can be performed under real-time image guidance, without the need for ureteral catheterization. It is hoped that such a technology will help reduce the risk of ureteral injury, although if an injury does occur, the repair can also be performed under direct visualization.⁹

The limitation of this study is that we are unable to predict whether our results in pigs can be generalized to humans. The sub-retroperitoneal layer in a pig is not as thick as in an adult human (Figure 3). Additionally, the ascending and descending colon are free from the retroperitoneum in pigs, while in humans they are located behind the retroperitoneum and are usually embedded in fat; thus, an extra dissection and a higher fluence rate of fluorescence excitation light might be needed. Although poor renal function might limit the utility of this technique, dilute MB in saline could always be infused retrograde from the bladder in order to highlight the ureters. However, preoperative ureteral catheterization has its own risk of ureteral

injury. We also encountered an unexpected problem in pigs—the high NIR autofluorescence in feces due to metabolites in their feed. What, if any, NIR fluorescence background there is in the human colon due to fecal remnants remains to be determined. Finally, although MB is known to cause false elevation in methemoglobin levels measured by CO-oximetry and pulse oximetry,²² we observed, at most, a 5% change in SpO₂ measurements during MB infusion. To minimize the likelihood of toxicity, it is prudent to use the lowest possible dose of MB needed for a particular case. And, it is important to exclude subjects with known glucose-6-phosphate dehydrogenase deficiency (G6PD), due to the risk of severe hemolysis.²³

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Abbreviations

2D	two-dimensional
3D	three-dimensional
BG	background
CBR	contrast-to-background
FDA	US Food and Drug Administration
FLARE™	Fluorescence Assisted Resection and Exploration image-guided surgery system
FI	fluorescence intensity
IV	intravenous
IVP	intravenous pyelography
LED	light emitting diode
LMB	leucomethylene blue
MB	methylene blue
NIR	near-infrared
PBS	phosphate-buffered saline
QY	quantum yield
ROI	region of interest
Uro-CT	urological computed tomography

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A.

Leucomethylene Blue (LMB)
MW 285.4

B.

Figure 1. Chemical Structures and Optical Properties of Methylene Blue (MB) and Leucomethylene Blue (LMB)

A. The chemical structures and redox coupling of MB (top; blue color) and LMB (bottom; colorless) is shown at left. Optical properties (right), including absorbance (Abs; solid curve) and fluorescence (FI; dashed curve), in pH-adjusted buffers as described in Materials and Methods.

B. Quenching (concentration vs. fluorescence) curve for MB in PBS, pH 7.4 (solid circles) or swine urine (open circles). Arrow indicates start of visible blue color to the solutions (≈ 15 μM), which increases with concentration.

Figure 2. Real-Time Intraoperative Near-Infrared Fluorescence Imaging Systems for Open and Laparoscopic Surgeries
A. The optical paths, dichroic mirrors (Dx), and filtration for the FLARE™ image-guided open surgery system used in this study. Filter wavelength ranges (nm) are provided for all excitation and emission filters.
B. Light paths and system components (left), and the actual apparatus (right), for the image-guided laparoscopy system used in this study.

Figure 3. NIR Fluorescence-Guided Intraoperative Identification of the Ureter

Shown are the color video (left column), NIR fluorescence (middle column), and a pseudo-colored (lime green) merged image of the two (right column). Exposure time was 150 msec for all NIR fluorescence images. Time post-injection of 0.1 mg/kg MB is indicated. K = kidney. Arrows = upper ureter. Arrowheads = lower ureter.

A.

B.

C.

Figure 4. Quantitative Assessment of Dose-Response and the Effect of Diuretics on MB NIR Fluorescence-Guided Ureteral Imaging

A. CBR (mean $\pm$ SEM) dose-response of 0.1 vs. 0.5 mg/kg MB in kidney (top), the upper ureter (middle), and the lower ureter (bottom) over time. Asterisks indicate $p < 0.05$. B.

CBR (mean $\pm$ SEM) of 0.1 mg/kg MB over time in kidney (top), the upper ureter (middle), and the lower ureter (bottom), with and without pre-injection of 10 mg furosemide.

C. Urine concentration (mean $\pm$ SEM) of MB after intravenous injection. Asterisks indicate $p < 0.05$.

Figure 5. Laparoscopic, NIR Fluorescence-Guided Ureteral Imaging using Methylene Blue
Shown are the color video (left), NIR fluorescence (middle), and a pseudo-colored (lime green) merged image of the two (right) 10 min after intravenous injection of 1 mg/kg MB. Arrows = ureter. The camera exposure time was 500 msec for NIR fluorescence images. K, kidney; Cr, cranial side; Ca; caudal side.

Table 1

Physiological Characteristics for Each Group [Mean (Range)].

	0.1 mg/kg MB (n = 8)	0.1 mg/kg MB + Furosemide (n = 6)	0.5 mg/kg (n = 6)	P value
Body weight (kg)	37.1 (33.4 – 40.3)	36.5 (32.9 – 39.1)	36.4 (31.8 – 40.1)	0.891
Urine pH				
Mean	6.25 (5.68 – 7.46)	6.31 (5.68 – 6.80)	6.49 (6.04 – 6.94)	0.674
Maximum	6.69 (6.14 – 7.65)	6.70 (6.37 – 6.92)	6.85 (6.39 – 7.79)	0.784
Minimum	5.92 (5.11 – 7.33)	6.08 (5.40 – 6.61)	6.37 (5.68 – 7.40)	0.433
Blood pH				
Mean	7.61 (7.48 – 7.75)	7.58 (7.42 – 7.65)	7.50 (7.24 – 7.65)	0.183
Maximum	7.66 (7.52 – 7.80)	7.64 (7.48 – 7.71)	7.60 (7.51 – 7.70)	0.473
Minimum	7.56 (7.37 – 7.69)	7.53 (7.37 – 7.64)	7.51 (7.41 – 7.62)	0.621
Baseline Urine Absorbance	2.70 (2.62 – 2.79)	2.68 (2.62 – 2.80)	2.75 (2.64 – 2.85)	0.257