INVESTIGATION OF BOWEL PERFORATION DETECTION USING FLUORESCENT CHLOROPHYLL DERIVATIVES

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Abstract- Chlorophyll derivatives that abundantly exist in food plants show bright emission bands at long wavelength regime (>650 nm) than those of auto-fluorescence of bio-tissues or -organs. In bowel perforation, intestinal fluids, which show bright and long wavelength regime emissions of chlorophyll derivatives, may leak from perforation sites. In order to examine a feasibility of fluorescence spectroscopy to be utilized as a real-time bio-sensor without using contrast agents for monitoring bowel perforation, we comparatively analyzed fluorescence obtained from intestinal and peritoneal fluids of mouse and rat models administered with alfalfa free feed known to minimize auto-fluorescence and improve imaging clarity. In addition, we analyzed samples from human patients who underwent surgery for small bowel resection and checked sensitivities of the device with various concentrations of intestinal fluids in peritoneal fluids. Fluorescence measurements were conducted using a portable optical-fiber-coupled fluorescence spectrometer equipped with Nd:YAG laser operating at 532 nm and photodiode-array-typed spectral analyzer. Compared to bare peritoneal fluids and bio-tissues or –organs, perforated intestine sites contaminated with intestinal contents show brighter (increased in intensity by factor of >8) and long wavelength regime fluorescence bands at ~680 and ~720 nm, which are assigned to fluorescence bands of pheophytin (chlorophyll derivative).

Keywords- Bowel perforation, Chlorophyll, Fluorescence spectroscopy.

I. INTRODUCTION

Bowel perforation is an emergency situation and causes infectious peritonitis (an inflammation of peritoneum induced by microorganisms) [1, 2]. Bowel perforation is caused by various factors such as peptic ulcer, inflammatory bowel disease, tumor, trauma, diverticulosis, swallowing of a foreign body and iatrogenic cases [3, 4]. Peritonitis is a life threatening situation and thus needs to be immediately treated [5].

For diagnosing bowel perforation, computational tomography (CT) imaging with intravenous radiocontrast medium has been widely used. However, CT requires movement of a patient to CT room and exposure of radiation. CT also needs use of radiocontrast agents, which may induce severe adverse effects such as life threatening anaphylactoid reaction, cardiovascular reaction, contrast induced nephropathy, and extravasation of contrast material [6, 7]. If a real time monitoring system that can be used in real operation room for monitoring bowel perforation without using radiation and contrast medium is developed, it will be great help to the surgeon and patient.

Food constituents and biologic tissues contain various auto-fluorescence materials [8, 9]. Most of auto-fluorescence materials including collagen, amino acid, enzyme, and coenzyme show emission bands at short wavelengths (at <650 nm), regardless of excitation wavelength (Table 1). Chlorophyll a consists of four pyrrole rings, phytol chain, and central magnesium ion (Fig. 1a). In heat and acid condition such as cooking, use of vinegar, and intragastric state after ingestion, chlorophyll loses magnesium ion and is converted to pheophytin. Fermented foods are also known to contain pheophorbide a, which is a molecule that loses long hydrocarbon chain from pheophyin [10]. Chlorophyll, pheophytin, and pheophorbide a consistently show intense fluorescence bands at ~680 nm, because of tetrapyrrole rings (Fig. 1b). We note that emission bands at >650 nm is unusual in the auto-fluorescence of biological tissues of internal organs.

When bowel perforation happens, intestinal fluids containing various auto-fluorescence materials may leak from perforated sites. In particular, chlorophylls that abundantly exist in food plants may show distinguishable fluorescence bands at long wavelength regime of >650 nm. Fluorescence quantum yield of chlorophyll a is known to be 0.23 in methanol solvent, which is much higher than those of general biologic tissues [11].

Peritoneal fluid is a composite of organic fluids that act as lubricant for visceral movement of organs in abdominal cavity and its volume in normal condition is known to be less than 5 ml for male and 5-18 ml for female, which depends on menstrual cycle [12, 13]. Peritoneal fluid contains several kinds of proteins such as albumin, immunoglobulin, and other electrolytes [14]. In normal condition, peritoneal fluids might not contain chlorophyll or its derivatives emitting at long wavelength regime of >650 nm. If bowel perforation happens, intestinal fluids containing chlorophylls may leak from perforation sites and be mixed with peritoneal fluids.

Inflammation caused by bowel perforation is also known to increase the volume of peritoneal fluid.

We here hypothesize that a bowel perforation may be detected without using radiocontrast agents by monitoring of peritoneal fluids containing chlorophylls. We use the excitation wavelength of 532 nm, because the excitation wavelength of shorter than 450 nm was found to excite various biologic tissues, which makes difficulty for discriminating fluorescence of chlorophyll derivatives, even though a sharp absorption band at ~400 nm (Soret band) leads to strong fluorescence of pheophytin (Fig. 1b, Table 1). The purpose of this study is to figure out a feasibility of chlorophyll fluorescence to be used as bio-marker for monitoring bowel perforation, despites of mixing of intestinal and peritoneal fluids. Our experiments were conducted for rats and humans.

II. DETAILS EXPERIMENTAL

2.1. Sample preparation

All the experiments conducted for animals were permitted from the Animal Care Committee of Gwangju Institute of Science and Technology (GIST2015-05). 6-8 Weeks old Balb/c female mouse and female Sprague Dawley rat (Orient Bio Inc., Gyeonggi-do, Korea) were used. Anesthesia was conducted using ketamine (70 mg/kg, Yuhan Ketamine 50, Yuhan Co., Ltd, Seoul, Korea) and xylazine (7 mg/kg, Bayer Korea, Gyeonggi-do, Korea). Wide incision was made for exposing intestines. 500 µl of 0.9% NaCl solution were injected to the peritoneal cavity through puncture of abdominal wall and aspirated after gentle manipulation for obtaining intestinal fluids. Abdominal wall with anterior peritoneum of animal was removed to measure in vivo fluorescence spectra. For bowel perforation, designated site of the bowel was perforated. Standard compounds including chlorophyll a, pheophytin, and pheophorbide a were purchased from Sigma-Aldrich, St Louis, MO.

For experiments using human samples, approval was made from the Chonnam National University Hospital Institutional Review Board (CNUH-2015-178). Intestinal fluids were acquired from the patient who underwent bowel resection surgery. Peritoneal fluids were obtained from the patient who underwent intra-abdominal surgery other than bowel resection. The fluids in drained bag after abdominal surgery other than bowel resection were regarded as peritoneal fluids. Intestinal fluids were systematically diluted with peritoneal fluids for determining the detection sensitivity of bowel perforation.

Table1: Auto-fluorescence of chlorophyll derivatives and biologic molecules

Molecule	Excitation (nm)	Fluorescence (nm)	Source	References
Chlorophyll	465, 665	660, 674, 726	Plants,	[15, 16]
Pheophytin	420-425, 615-655	660, 674	Plants	[16]
Pheophorbide	615-655	660, 674	Plants	[16]
NAD(P)H	340	450	All	[17]
Collagen	270-370	305-450	Animals	[17]
Retinol		500	Animals & bacteria	[18]
Riboflavin		550	All	[18]
Cholecalciferol		380-460	Animals	[18]
Folic acid		450	All	[18]
Pyridoxine		400	All	[18]
Tyrosine	270	305	All	[19]
Dityrosine	325	400	Animals	[19]
Glycation adduct	370	450	Animals	[19]
Tryptophan	280	300-350	All	[20]
Flavin	380-490	520-560	All	[21]
Melanin	340–400	360–560	Animals	[22]

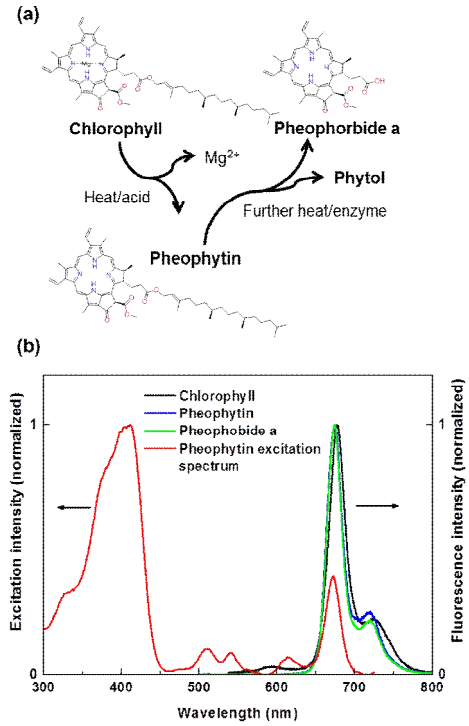

Fig.1. (a) Metabolism of chlorophyll a in acid and heat condition, (b) excitation spectrum of pheophytin and emission spectra of chlorophyll derivatives

2.2. IVIS fluorescence image measurement

IVIS (In vivo imaging system, Xenogen, USA) was used for obtaining fluorescence images for rat and mouse organs. Intestinal fluids leaking from perforation sites were visualized by measurement of IVIS images. The excitation wavelength of 535 nm was used for IVIS image measruements. After removal of abdominal wall including anterior peritoneum, IVIS images were measured.

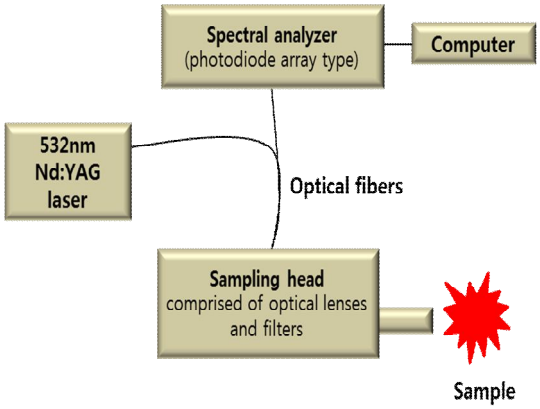

Fig.2. Schematic diagram of the experiment set up.

2.3. Fluorescence spectrum measurement

Fluorescence spectra of peritoneal fluid, intestinal fluid, and rat internal organs were measured using a home-made portable fluorescence spectrometer. Light source was Nd:YAG laser operating at 532 nm. The excitation light (Nd-YAG laser beam) was focused on the sample after passing through optical fiber, band pass filter, and a focus lens. Emitted fluorescence lights were transferred to a photodiode-array-typed spectral analyzer after passing through collection lens and long wavelength pass filter. Fluorescence spectra were instantaneously displayed on computer screen (Fig. 2)

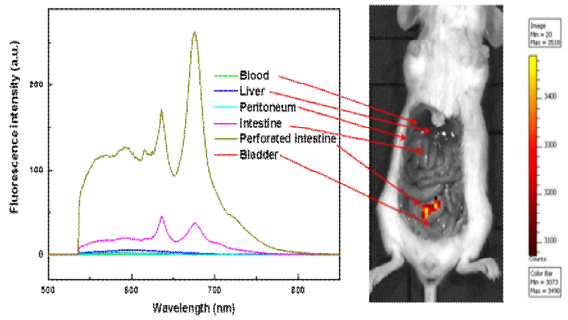

Fig.3. Fluorescence spectra of internal mouse organs measured using a portable fluorescence spectrometer (left). IVIS image for mouse measured using 535 nm excitation and 680 nm emission wavelengths and optimizing IVIS signal thresholds (right)

III. RESULTS AND DISCUSSION

3.1. Fluorescence of animal internal organs and perforated bowel

Bowel consists of stomach, small intestine (duodenum, jejunum, ilium, cecum, and appendix), and large intestine (ascending colon, transverse colon, descending colon, sigmoid colon, and rectum). Figure 3 shows the fluorescence spectra corresponding to liver, peritoneum, bladder, intestine, and perforated intestine of mouse. Because the developed fluorescence spectrometer uses lens for focusing the excitation light and collecting the emission lights, optical alignment should be adjusted for each measurement. Intestines show intense fluorescence bands at ~680 nm, consistent with fluorescence band of chlorophyll derivatives (see Figs. 1 and 3). Blood, bladder, liver, and peritoneum show very weak fluorescence bands, compared to those of intestines and perforated intestines.

IVIS measurements show intense fluorescence signals at perforated sites (Fig. 3) when using the excitation and emission wavelengths of 535 and 680 nm, respectively. Intestinal fluids containing chlorophylls may spread to adjacent area after bowel perforation. Perforated sites shows intense fluorescence signals at 680 nm, indicating that intestinal fluids leaking from perforated intestine sites are successfully monitored by measurements of fluorescence signals at 680 nm (chlorophyll emission regime). This finding demonstrates a potential of chlorophylls to be utilized as a biomarker for monitoring bowel perforation.

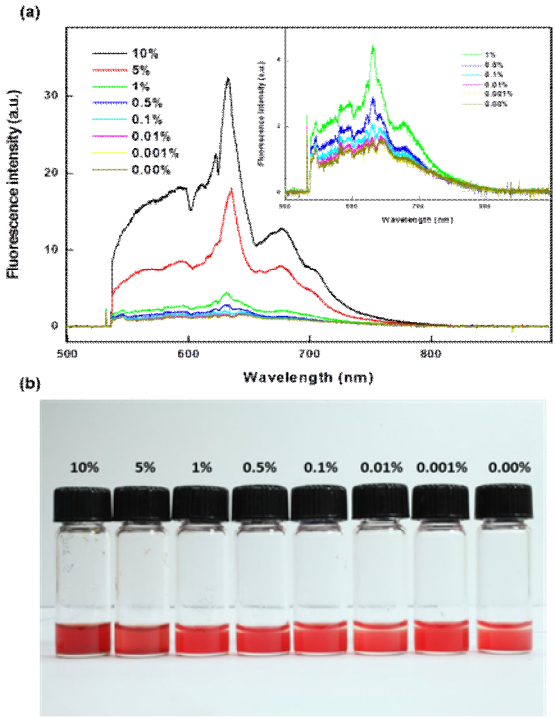

Fig.4. (a) Fluorescence spectra of rat intestinal fluids diluted with peritoneal fluids, (b) photo of the sample solutions. Intestinal fluids are systematically diluted with peritoneal fluids for showing various concentrations, as indicated.

3.2. Detection of composites of intestinal and peritoneal fluids of rat and human

In bowel perforation, a few volumes of intestinal fluids may leak from perforation sites and be mixed with peritoneal fluids. Following this hypothesis, we arbitrary mixed intestinal and peritoneal fluids of rat. Figure 4a shows fluorescence spectra of intestinal fluids systematically diluted with peritoneal fluids. The peaks at ~640 and ~680 nm are assigned to fluorescence bands of chlorophylls. The samples with concentrations of 10, 5, and 1% show manifested fluorescence bands at ~640 and ~680 nm, compared to that of bare peritoneal fluid. The samples with concentrations of 0.5 and 0.1% show distinguishable fluorescence bands at ~640 and ~680 nm, compared to those found for 0.01, 0.001, and 0.00%. Figure 5b

shows real appearance of the sample solutions. The red color were found because the peritoneal fluids of rat were mixed with bloods. Note that the 10 and 5% solutions show darker colors than those for diluted solutions. The samples with concentrations of less than 1% don not show any color differences when monitored with human eyes.

Figure 5a shows that fluorescence spectra of human intestinal fluids systematically diluted with pure peritoneal fluids. The sample with 100% intestinal fluid obtained from ileum after bowel resection shows intense fluorescence band at ~680 nm, consistent with fluorescence band of chlorophylls. This band was manifestly found for 10, 5, and 1% solutions, but not for 0.1, 0.01, 0.00% solutions. The peaks found for the samples with concentration of less than 0.1% make hard to discriminate the fluorescence of intestinal and peritoneal fluids. The sample with concentration of 100% intestinal fluid shows dark green color with high viscosity (Fig. 5b). This green color may come from bile juice of gall bladder and/or ingested food plants. The samples with concentration of 10 and 5% show green colors, which make us distinguish intestinal fluids from peritoneal fluids. However, the samples at concentration of less than 1% show similar yellowish colors. In real-field operation, the detection limit of surgeon's visual inspection for monitoring mixtures of intestinal and peritoneal fluids may be higher than 1%. However, the detection limit can be lower to the range of 0.1-1% by using our fluorescence detection method.

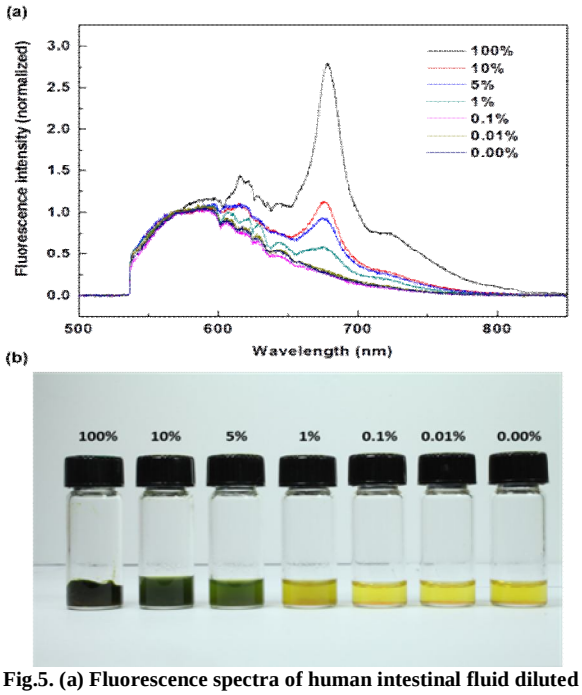

Fig.5. (a) Fluorescence spectra of human intestinal fluid diluted with peritoneal fluids, (b) photos of the sample solutions

3.3. Point of view in medical field

Because the fluorescence of intestinal fluids are different in wavelength and intensity from those of peritoneal fluids, a bowel perforation can be detected by monitoring fluorescence spectra of peritoneal fluids of suspicious patients. Intestinal fluids containing chlorophyll derivatives may leak from perforation sites because of intestinal pressure difference. Bowel perforation can be monitored in real time by instantaneous detection of long wavelength regime emissions (at >670 nm) of chlorophylls. By monitoring of the fluorescence at suction bottle or tip that collects or aspirates the fluids from abdominal cavity during the surgery, one can facilely monitor bowel perforation.

Recently, laparoscopic surgery is favored as a first approach of abdominal organ surgery. This minimally invasive surgery gives lower recovery burden to patients than classic surgery does. However, laparoscopic surgery met with difficulties in visual and haptic perceptions [23], and complications caused by other organ damage such as iatrogenic bowel perforation are occasionally reported. Incidence probability of bowel perforation during the laparoscopic surgery is reported as 0.2 to 0.22% [24, 25]. If our fluorescence detection device is combined with laparoscopic surgery and used for monitoring bowel perforation during abdominal surgery, it will be help to significantly reduce incidence probability.

When bowel perforation or intra-abdominal hemorrhages are suspicious, diagnostic peritoneal lavage (DPL) may be used. DPL is a detection method for bowel perforation, which is enabled by microscopic analysis of red blood cells, white blood cells, bile juice, food components, amylase, bilirubin, and other substituents of peritoneal fluids that are obtained by using aspiration through minimal incision of abdomen. Fluorescence detection method of chlorophylls demonstrated in this study may help to instantaneous detection of bowel perforation in operation room when using aspiration techniques developed for DPL method.

CONCLUSIONS

The fluorescence spectra found for intestinal fluids of rat and human containing chlorophyll derivatives show different profiles, compared to those of bare peritoneal fluids, when the intestinal fluids were diluted with pure peritoneal fluids up to 0.1 and 1%, respectively. Using these characteristics, one can safely monitor a bowel perforation without using radiation, radiocontrast, and CT room, reducing the burden of a surgeon and patient.

ACKNOWLEDGMENTS

This research was supported by the Advanced Photonics Research Institute (APRI) Research Program and the Ultrashort Quantum Beam Facility Program through a grant from the Gwangju Institute of Science and Technology in 2015. Financial support was also obtained through grant No. NRF-2014R1A1A2A10053439 from the National Research Foundation funded by the Korean Government

Ministry of Education, Science, and from Technology and (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2014R1A2A1A11052300) and Basic Research Projects in High-tech Industrial Technology Project through a grant provided by GIST in 2015.

REFERENCES

[1]. M. J. Baron, and D. L. Kasper, "Intraabdominal infections and abscesses," Harrison's principles of internal medicine. 18th ed. New York: McGraw-Hill, 1081 (2012).
[2]. T. Calandra, and J. Cohen, "The international sepsis forum consensus conference on definitions of infection in the intensive care unit," Critical care medicine 33, 1538-1548 (2005).
[3]. N. A. Kimchi, E. Broide, M. Shapiro, and E. Scapa, "Non-traumatic perforation of the small intestine. Report of 13 cases and review of the literature," Hepato-gastroenterology 49, 1017-1022 (2001).
[4]. A. Tridente, G. M. Clarke, A. Walden, S. McKechnie, P. Hutton, G. Mills, A. Gordon, P. Holloway, J.-D. Chiche, and J. Bion, "Patients with faecal peritonitis admitted to European intensive care units: an epidemiological survey of the GenOSept cohort," Intensive care medicine 40, 202-210 (2014).
[5]. E. H. Farthmann, and U. Schöffel, "Principles and limitations of operative management of intraabdominal infections," World journal of surgery 14, 210-217 (1990).
[6]. E. C. Lasser, C. C. Berry, L. B. Talner, L. C. Santini, E. K. Lang, F. H. Gerber, and H. O. Stolberg, "Pretreatment with corticosteroids to alleviate reactions to intravenous contrast material," New England Journal of Medicine 317, 845-849 (1987).
[7]. S. Morcos, "Acute serious and fatal reactions to contrast media: our current understanding," The British journal of radiology (2014).
[8]. L. Bachmann, D. M. Zezell, A. d. C. Ribeiro, L. Gomes, and A. S. Ito, "Fluorescence spectroscopy of biological tissues—A review," Applied Spectroscopy Reviews 41, 575-590 (2006).
[9]. R. Karoui, and C. Blecker, "Fluorescence spectroscopy measurement for quality assessment of food systems—a review," Food and Bioprocess technology 4, 364-386 (2011).
[10]. J. W. Heaton, and A. G. Marangoni, "Chlorophyll degradation in processed foods and senescent plant tissues," Trends in Food Science & Technology 7, 8-15 (1996).
[11]. G. Weber, and F. Teale, "Determination of the absolute quantum yield of fluorescent solutions," Transactions of the Faraday Society 53, 646-655 (1957).
[12]. B. Hills, "Lubrication of visceral movement and gastric motility by peritoneal surfactant," Journal of gastroenterology and hepatology 11, 797-803 (1996).
[13]. P. McGing, and R. O'Kelley, "The biochemistry of body fluids," Ireland: The Scientific Committee of the Association of Clinical Biochemists in Ireland (ACBI) (2009).
[14]. J. Kelton, R. Ulan, C. Stiller, and E. Holmes, "Comparison of chemical composition of peritoneal fluid and serum: a method for monitoring dialysis patients and a tool for assessing binding to serum proteins in vivo," Annals of internal medicine 89, 67-70 (1978).
[15]. M. Mimuro, S. Akimoto, I. Yamazaki, H. Miyashita, and S. Miyachi, "Fluorescence properties of chlorophyll d-dominating prokaryotic alga, Acaryochloris marina: studies using time-resolved fluorescence spectroscopy on intact cells," Biochimica et Biophysica Acta (BBA)-Bioenergetics 1412, 37-46 (1999).
[16]. M. B. Bazzaz, and C. A. Rebeiz, "Spectrofluorometric determination of chlorophyll (ide) a and b and pheophytin (or pheophorbide) a and b in unsegregated pigment mixtures," Photochemistry and Photobiology 30, 709-721 (1979).
[17]. I. Georgakoudi, B. C. Jacobson, M. G. Müller, E. E. Sheets, K. Badizadegan, D. L. Carr-Locke, C. P. Crum, C. W. Boone, R. R. Dasari, and J. Van Dam, "NAD (P) H and collagen as in vivo quantitative fluorescent biomarkers of epithelial precancerous changes," Cancer research 62, 682-687 (2002).
[18]. W. R. Zipfel, R. M. Williams, R. Christie, A. Y. Nikitin, B. T. Hyman, and W. W. Webb, "Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation," Proceedings of the National Academy of Sciences 100, 7075-7080 (2003).
[19]. J. M. Menter, "Temperature dependence of collagen fluorescence," Photochemical & Photobiological Sciences 5, 403-410 (2006).
[20]. L. Brancaleon, G. Lin, and N. Kollias, "The in vivo fluorescence of tryptophan moieties in human skin increases with UV exposure and is a marker for epidermal proliferation," Journal of investigative dermatology 113, 977-982 (1999).
[21]. D. Chorvat, J. Kirchnerova, M. Cagalinec, J. Smolka, A. Mateasik, and A. Chorvatova, "Spectral unmixing of flavin autofluorescence components in cardiac myocytes," Biophysical journal 89, L55-L57 (2005).
[22]. J. M. Gallas, and M. Eisner, "Fluorescence of melanin - Dependence upton excitation wavelength and concentration," Photochemistry and photobiology 45, 595-600 (1987).
[23]. H. Xin, J. Zelek, and H. Carnahan, "Laparoscopic surgery, perceptual limitations and force: A review," in First Canadian student conference on biomedical computing(Citeseer, 2006).
[24]. J. T. Bishoff, M. E. ALLAF, W. Kirkels, R. G. Moore, L. R. KAVOUSSI, and F. Schroder, "Laparoscopic bowel injury: incidence and clinical presentation," The Journal of urology 161, 887-890 (1999).
[25]. M. Van der Voort, E. Heijnsdijk, and D. Gouma, "Bowel injury as a complication of laparoscopy," British journal of surgery 91, 1253-1258 (2004).

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