Heliyon

ELSEVIER

Received:
7 April 2016
Revised:
6 July 2016
Accepted:
2 August 2016

Heliyon 2 (2016) e00139

Fluorescence lifetime measurement with confocal endomicroscopy for direct analysis of tissue biochemistry *in vivo*

Youngjae Won [a,1], **Byungjun Park** [a,1], **Inwook Kim** [b], **Seungrag Lee** [a,*]

[a] *Medical Device Development Center, Osong Medical Innovation Foundation, Cheongju, Chungbuk, 28160, Republic of Korea*

[b] *Catholic Research Institute of Medical Science, The Catholic University of Korea, Seoul, 137-071, Republic of Korea*

* Corresponding author.

E-mail address: naviman78@gmail.com (S. Lee).

[1] These authors contributed equally to this work.

Abstract

Confocal endomicroscopy is a powerful tool for *in vivo* real-time imaging at cellular resolution inside a living body without tissue resection. Microscopic fluorescence lifetime measurement can provide information about localized biochemical conditions such as pH and the concentrations of oxygen and calcium. We hypothesized that combining these techniques could assist accurate cancer discrimination by providing both biochemical and morphological information. We designed a dual-mode experimental setup for confocal endomicroscopic imaging and fluorescence lifetime measurement and applied it to a mouse xenograft model of activated human pancreatic cancer generated by subcutaneous injection of AsPC-1 tumor cells. Using this method with pH-sensitive sodium fluorescein injection, we demonstrated discrimination between normal and cancerous tissues in a living mouse. With further development, this method may be useful for clinical cancer detection.

Keywords: Biochemistry, Bioengineering, Biophysics, Biotechnology, Cell biology, Engineering

http://dx.doi.org/10.1016/j.heliyon.2016.e00139

1. Introduction

There has been a growing interest in diagnosing cancer without tissue resection using a minimally invasive and fast approach that can replace immunohistochemical staining [1, 2, 3, 4]. Confocal endomicroscopy is a FDA-approved diagnostic tool that enables the minimally invasive discrimination of abnormal lesions from normal tissue surfaces inside a living body by high-resolution cellular imaging. This technique has the advantage that optical biopsy images are displayed in real-time to assist the physician in making immediate patient management decisions. Many studies have demonstrated the use of confocal endomicroscopy for cancer detection in various bodily regions, including stomach, pancreas, colon, alveoli, bladder, and others [5, 6, 7, 8, 9, 10]. Despite its powerful advantages, several issues have prevented confocal endomicroscopy from becoming widely accepted in clinical use [11]. In particular, diagnostic efficacy is the most important area in which its reliability has not yet been adequately validated.

The fluorescence lifetime of a molecule is the average time that it spends in the excited state after absorbing extremely short pulsed laser energy [12]. Molecular fluorescence lifetime is not dependent on laser power, the concentration of the molecule, or photo-bleaching, but varies only due to the influence of the local biochemical conditions on energy transfer. Therefore, fluorescence lifetime can be used as an indicator of local biochemical conditions such as pH, the concentrations of oxygen and calcium, and other factors [12, 13, 14, 15]. Various fluorescence dyes have been used for *in vivo* and *ex vivo* studies of local biochemical conditions to identify abnormal cells or tissues [12]. To avoid side effects of fluorescence dyes, auto-fluorescence naturally emitted by endogenous fluorophores in living tissues can also be applied for fluorescence lifetime measurements [16]. Certain cancer-related auto-fluorescent molecules such as nicotinamide adenine dinucleotide, flavin, and collagen may have altered fluorescence lifetimes due to local perturbations in oxygen concentration and pH. Thus, fluorescent lifetime measurement of auto-fluorescent molecules might present a foundation for the detection of abnormal tissues. The functional information that can be obtained by fluorescence lifetime measurements may help to make confocal endomicroscopy a reliable diagnostic tool for cancer detection.

In this study, we designed a dual-mode experimental setup for confocal endomicroscopic imaging and fluorescence lifetime measurement. A commercially available confocal endomicroscope (Cellvizio, Mauna Kea Technologies, Paris, France) and fluorescence lifetime spectrometer (FluoTime 300, PicoQuant, Berlin, Germany) were linked to the same optical mini-probe (GastroFlex UHD, Mauna Kea Technologies, Paris, France). To evaluate the effectiveness of this approach for cancer detection, we subcutaneously injected AsPC-1 activated human pancreatic cancer cells into a living mouse, followed by intravenous injection of

http://dx.doi.org/10.1016/j.heliyon.2016.e00139

sodium fluorescein. Sodium fluorescein is a FDA-approved fluorophore widely used as a diagnostic tool in the fields of ophthalmology and cancer detection. The fluorescence lifetime of sodium fluorescein depends on the local pH [17]. Since pH differs between abnormal and normal tissues, they can be differentiated by measuring the fluorescence lifetime of pH-sensitive sodium fluorescein. We morphologically discriminated abnormal and normal tissues by confocal endomicroscopic imaging, and then functionally discriminated them by measuring the fluorescence lifetime of injected sodium fluorescein.

2. Material and methods

2.1. Experimental setup

An experimental concept for the analysis of local biochemical conditions by fluorescence lifetime measurement combined with confocal endomicroscopy is presented in Fig. 1. Nude mouse activated human pancreas cancer AsPC-1 was prepared for the animal test. Sodium fluorescein was used as a fluorescent probe and intravenously injected into the mouse model. Then, the living mouse tissues were morphologically screened by confocal endomicroscopy. When tissues with an unusual morphology were identified by confocal imaging, the distal end of the probe was fixed using a holding jig and the opposite end of the probe was removed from confocal endomicroscopy and connected to the interface module for measurement of fluorescence lifetime. The fluorescence lifetime of sodium fluorescein diffused in that area was measured by fluorescence lifetime spectroscopy. The same process was applied for normal tissue.

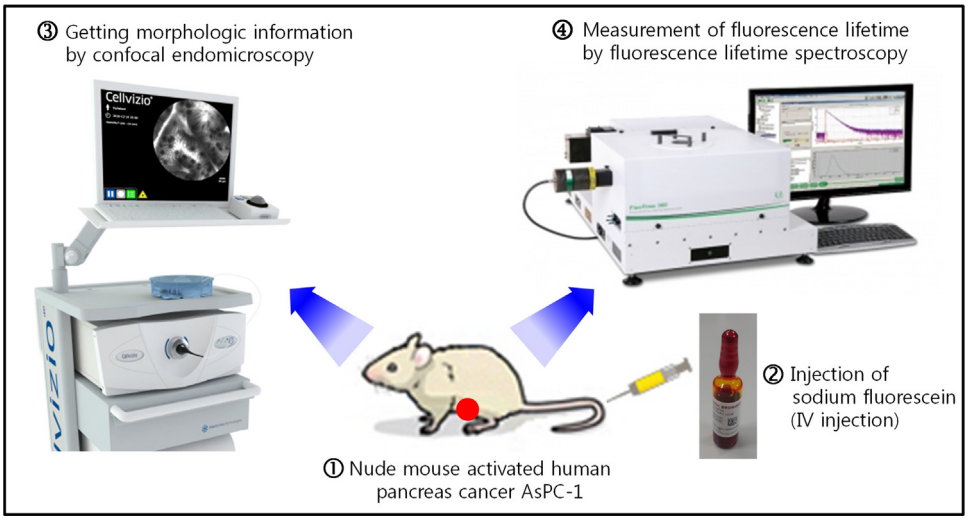


Fig. 1. Experimental concept: (1) Preparation of nude mouse activated human pancreas cancer AsPC-1 (2) Injection of sodium fluorescein (Intravenous (IV) injection) (3) Getting morphologic information by confocal endomicroscopy (4) Measurement of fluorescence lifetime by fluorescence lifetime spectroscopy.

http://dx.doi.org/10.1016/j.heliyon.2016.e00139

The dual-mode experimental setup for confocal fluorescence endomicroscopic imaging and fluorescence lifetime measurement was shown in Fig. 2. After generating the mouse model, it was morphologically analyzed by confocal endomicroscopy with confocal mini-probe consisted of about 30,000 optical fibers. The diameter and working distance of used mini-probe was about 2.8 mm and 55–66 um, respectively. The confocal endomicroscopy provided the image as a circular pattern. The diameter for maximum field of view was 600 um and the lateral resolution was 3.5 um. The used light source of the confocal endomicroscopy was continuous laser with a center wavelength (λc) of 488 nm and the average incident power (Pavg) of 1 mW. After morphological analysis, the same mini-probe used for confocal endomicroscopy was applied as a probe for fluorescence lifetime measurements. The fluorescence lifetime of the same region examined by confocal imaging could be measured by fixing the distal end of the mini-probe and moving its opposite end to the specially designed optical interface module. The optical interface module was realized by confocal point measurement setup to perform confocal fluorescence lifetime measurement. For the light source of fluorescence lifetime spectrometer, a diode pulse laser with a center wavelength of 479 nm, the average incident power (Pavg) of 2 mW, the pulse duration (frr) of 10 MHz, and the pulse width (△λ) of 30 ps was used. The diode pulse laser was passed through a short pass filter with cutoff wavelength of 500 nm and reflected by a dichroic mirror and injected into the region of interest of the living mouse after passing through an objective lens and the mini-probe. The fluorescence pulse signals from fluorescein inside the mouse were transmitted through the same components in the reverse direction and passed through the dichroic mirror, a long pass filter with cutoff wavelength of 500 nm, and collimating lens. Finally, the

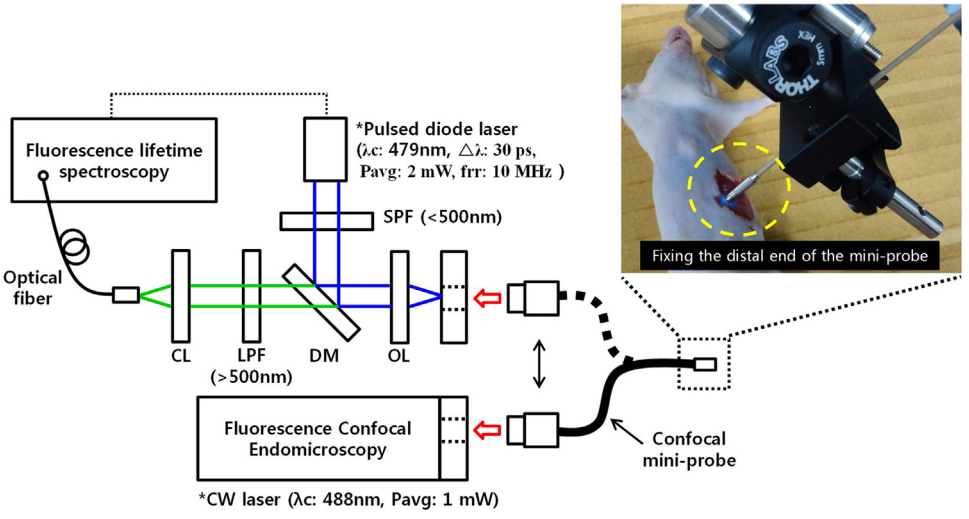


Fig. 2. Dual-mode experimental setup for confocal fluorescence endomicroscopic imaging and fluorescence lifetime measurement.

http://dx.doi.org/10.1016/j.heliyon.2016.e00139

fluorescence pulse signals went to the optical fiber of the fluorescence lifetime spectrometer.

2.2. Preparation of animal model

In vivo tumor xenograft experiments were conducted using 6-week-old male athymic nude mouse (BALB/c-nu) purchased from Central Lab. Animal Inc. (Seoul, South Korea). As shown in Fig. 3, tumors were implanted into the abdomens of the mouse by the subcutaneous injection of 1×10^7 AsPC-1 human pancreas cancer cells in 100 μL of serum-free Dulbecco's modified Eagle's medium (Gibco®, Thermo Fisher Scientific, Waltham, MA). The mouse was maintained in a laminar air flow cabinet under specific pathogen-free conditions. Ethical approval was received and all facilities were approved by the Association of Assessment and Accreditation of Laboratory Animal Care, and all animal experiments were conducted under the institutional guidelines established by the Animal Core Facility at The Catholic University of Korea. This animal care and use protocol was reviewed and approved by the IACUC in School of Medicine, The Catholic University of Korea (CUMC-2015-0189-02). When the tumor size reached 100–150 mm^3, fluorescein was injected intravenously via the tail vein. Sodium fluorescein (100 mg/mL, 10%;[w/v], Novartis, Basel, Switzerland) was diluted to a final concentration of 0.01% (w/v) with phosphate buffered saline, and 200 μl of the diluted solution was injected into the mouse tail vein.

3. Results and discussion

To detect local pH variation between abnormal and normal tissues in a living mouse, sodium fluorescein was injected as described above, and the abnormal and normal tissues of the mouse were screened using standard confocal endomicroscopy. It has been demonstrated that morphological changes such as fibrosis often occur in tissues associated with neoplasia [11]. For confocal endomicroscopy, there is a library of case studies and atlases of normal and disease states available online, which we used for reference [18]. Through confocal fluorescence endomicroscopy analysis, we identified morphological differences between the

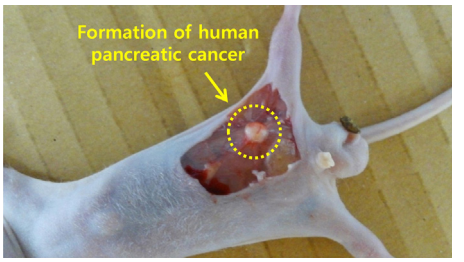


Fig. 3. Formation of human pancreatic cancer in a mouse model following subcutaneous injection of AsPC-1 cells.

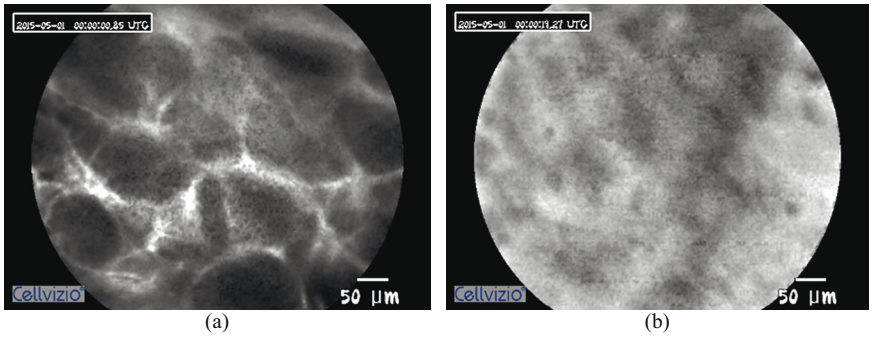
http://dx.doi.org/10.1016/j.heliyon.2016.e00139

cancerous and normal tissues as shown in Fig. 4. Confocal fluorescence images of normal mouse abdominal subcutaneous tissue and the same tissue containing structures associated with the pancreatic cancer structures are shown in Fig. 4(a) and (b), respectively. Here, the normal mouse abdominal subcutaneous tissue architecture is visualized well on a cellular level while pancreatic cancer tissue is not clear [19].

To discriminate abnormal tissues from normal tissues, the fluorescence lifetime of injected pH-sensitive sodium fluorescein was measured by fluorescence lifetime spectroscopy. Fluorescence curves after excitation by laser pulses were measured and plotted as shown in Fig. 5. Since the fluorescence lifetime of sodium fluorescein decreased according to the drop in pH, the widths of the fluorescence curves were shorter in abnormal than in normal tissue, indicating that the pH value of abnormal tissue was lower than that of normal tissue [17]. Curve fitting and calculation of fluorescence lifetimes were performed with FluoFit software (PicoQuant). Fluorescence curves were measured for three different areas in each of the normal and abnormal tissues. The photon numbers used to plot the fluorescence curves were more than 100,000. The measured fluorescence lifetimes of sodium fluorescein inside the normal and abnormal tissues were 3.69 ~ 3.81 and 3.09 ~ 3.44 ns, respectively. Since the measured fluorescence lifetimes for abnormal tissues were well differentiated from those for normal tissues, the fluorescence lifetime of sodium fluorescein could be used as an indicator to increase the accuracy of cancer detection with confocal endomicroscopy.

4. Conclusions

In this paper, we have demonstrated the application of fluorescence lifetime measurement combined with confocal endomicroscopy for the analysis of tissue biochemistry in a living mouse xenograft model of activated human pancreatic cancer. The abnormal and normal tissues were morphologically analyzed by confocal microendoscopic imaging, then we evaluated the possibility of



(a) (b)

Fig. 4. Confocal fluorescence imaging of (a) normal and (b) pancreatic cancer infiltrated mouse abdominal subcutaneous tissue.

http://dx.doi.org/10.1016/j.heliyon.2016.e00139

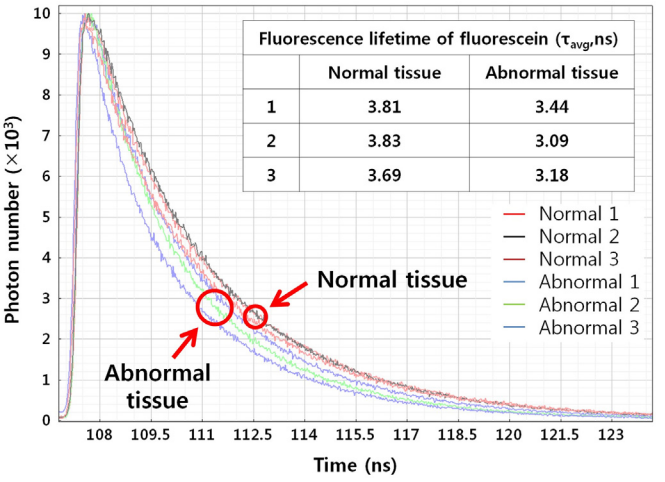


Fig. 5. Confocal fluorescence lifetime of fluorescein in (a) normal and (b) abnormal tissues screened by confocal endomicroscopy.

discriminating between them based on the fluorescence lifetime of sodium fluorescein, which is mainly influenced by the local tissue pH. We successfully discriminated the cancerous and normal tissues, indicating that this method may enable early cancer detection without tissue resection. Subsequent studies are warranted to assess the utility of applying this technique for fluorescent lifetime measurements of endogenous autofluorescent materials or other biocompatible fluorescent materials that are sensitive to changes in parameters such as pH, oxygen concentration, and temperature in the cancer tissue environment.

Declarations

Author contribution statement

Youngjae Won: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Byungjun Park, Inwook Kim: Performed the experiments; Analyzed and interpreted the data.

Seungrag Lee: Conceived and designed the experiments; Wrote the paper.

Funding statement

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2015R1C1A1A02036475).

http://dx.doi.org/10.1016/j.heliyon.2016.e00139

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

[1] R. Shukla, W.M. Abidi, R. Richards-Kortum, S. Anandasabapathy, Endoscopic imaging: How far are we from real-time histology? World J. Gastrointest. Endosc. 3 (2011) 183–194.

[2] S. Khondee, T.D. Wang, Progress in molecular imaging in endoscopy and endomicroscopy for cancer imaging, J. Healthc. Eng. 4 (2013) 1–21.

[3] S.F. Elahi, T.D. Wang, Future and advances in endoscopy, J. Biophotonics 4 (2011) 471–481.

[4] M.J. Bruno, Magnification endoscopy, high resolution endoscopy, and chromoscopy; towards a better optical diagnosis, Gut 52 (2003) iv7–iv11.

[5] Y.T. Guo, Y.Q. Li, T. Yu, T.G. Zhang, J.N. Zhang, H. Liu, F.G. Liu, X.J. Xie, Q. Zhu, Y.A. Zhao, Diagnosis of gastric intestinal metaplasia with confocal laser endomicroscopy *in vivo*: a prospective study, Endoscopy 40 (7) (2008) 547–553.

[6] M. Giovannini, F. Caillol, F. Poizat, E. Bories, C. Pesenti, G. Monges, J.L. Raoul, Feasibility of intratumoral confocal microscopy under endoscopic ultrasound guidance, Endosc. Ultrasound 1 (2) (2012) 80–83.

[7] M.W. Shahid, A. Buchner, V. Gomez, M. Krishna, T.A. Woodward, M. Raimondo, M.B. Wallace, Diagnostic accuracy of probe-based confocal laser endomicroscopy and narrow band imaging in detection of dysplasia in duodenal polyps, J. Clin. Gastroenterol. 46 (5) (2012) 382–389.

[8] F.S. Fuchs, S. Zirlik, K. Hildner, M. Frieser, M. Ganslmayer, S. Schwarz, M. Uder, M.F. Neurath, Fluorescein-aided confocal laser endomicroscopy of the lung, Respiration 81 (1) (2011) 32–38.

[9] J. Liu, J.J. Liu, W. Adams, G.A. Sonn, K.E. Mach, Y. Pan, A.H. Beck, K.C. Jensen, J.C. Liao, Dynamic real-time microscopy of the urinary tract using confocal laser endomicroscopy, Urology 78 (1) (2011) 225–231.

[10] P. Sharma, A.R. Meining, E. Coron, C.J. Lightdale, H.C. Wolfsen, A. Bansal, M. Bajbouj, J.P. Galmiche, J.A. Abrams, A. Rastogi, N. Gupta, J.E. Michalek, G.Y. Lauwers, M.B. Wallace, Real-time increased detection of

http://dx.doi.org/10.1016/j.heliyon.2016.e00139

neoplastic tissue in Barrett's esophagus with probe-based confocal laser endomicroscopy: final results of a multi-center prospective international randomized controlled trial, Gastrointest. Endosc. 74 (3) (2011) 465–472.

[11] S.S. Chauhan, B.K.A. Dayyeh, Y.M. Bhat, K.T. Gottlieb, J.H. Hwang, S. Komanduri, V. Konda, S.K. Lo, M.A. Manfredi, J.T. Maple, F.M. Murad, U. D. Siddiqui, S. Banerjee, M.B. Wallace, Confocal laser endomicroscopy, Gastrointest. Endosc. 80 (6) (2014) 928–938.

[12] H.C. Gerritsen, M.A. Asselbergs, A.V. Agronskaia, W.G. Van Sark, Fluorescence lifetime imaging in scanning microscopy, In: J.B. Pawley (Ed.), Handbook of Biological Confocal Microscopy, 3rd Edition, Springer, New York, 2006, pp. 516–534.

[13] H.J. Lin, P. Herman, J.R. Lakowicz, Fluorescence lifetime-resolved pH imaging of living cells, Cytometry A 52 (2) (2003) 77–89.

[14] H.C. Gerritsen, R. Sanders, A. Draaijer, C. Ince, Y.K. Levine, Fluorescence lifetime imaging of oxygen in living cells, J. Fluoresc. 7 (1) (1997) 11–15.

[15] A.V. Agronskaia, L. Tertoolen, H.C. Gerritsen, Fast fluorescence lifetime imaging of calcium in living cells, J. Biomed. Opt. 9 (6) (2004) 1230–1237.

[16] J. McGinty, N.P. Galletly, C. Dunsby, I. Munro, D.S. Elson, J. Requejo-Isidro, P. Cohen, R. Ahmad, A. Forsyth, A.V. Thillainayagam, M.A. Neil, P. M. French, G.W. Stamp, Wide-field fluorescence lifetime imaging of cancer, Biomed. Opt. Express 1 (2) (2010) 627–640.

[17] M. Hammer, D. Schweitzer, S. Richter, E. Königsdörffer, Sodium fluorescein as a retinal pH indicator? Physiol. Meas. 26 (2005) N9–N12.

[18] Muana Kea Technologies, Cellvizio confocal endomicroscopy case studies library and tissue atlas. http://www.cellvizio.net.

[19] S. Eser, M. Messer, P. Eser, A. von Werder, B. Seidler, M. Bajbouj, R. Vogelmann, A. Meining, J. von Burstin, H. Algul, P. Pagel, E. Angelika Schneike, I. Esposito, M. Roland Schmid, G. Schneider, D. Saur, In vivo diagnosis of murine pancreatic intraepithelial neoplasia and early-stage pancreatic cancer by molecular imaging, PNAS 108 (24) (2011) 9945–9950.

http://dx.doi.org/10.1016/j.heliyon.2016.e00139