Fluorescence lifetime imaging microscopy and its applications in skin cancer diagnosis

Lixin Liu*‡, Qianqian Yang*, Meiling Zhang*, Zhaoqing Wu* and Ping Xue†

*School of Physics and Optoelectronic Engineering
Xidian University, Xi’an 710071, P. R. China
†State Key Laboratory of Low-Dimensional Quantum Physics
Department of Physics, Tsinghua University
Beijing 100084, P. R. China
‡lxliu@xidian.edu.cn

Received 26 March 2019
Accepted 10 June 2019
Published 16 July 2019

Fluorescence lifetime (FLT) of fluorophores is sensitive to the changes in their surrounding microenvironment, and hence it can quantitatively reveal the physiological characterization of the tissue under investigation. Fluorescence lifetime imaging microscopy (FLIM) provides not only morphological but also functional information of the tissue by producing spatially resolved image of fluorophore lifetime, which can be used as a signature of disorder and/or malignancy in diseased tissues. In this paper, we begin by introducing the basic principle and common detection methods of FLIM. Then the recent advances in the FLIM-based diagnosis of three different skin cancers, including basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma (MM) are reviewed. Furthermore, the potential advantages of FLIM in skin cancer diagnosis and the challenges that may be faced in the future are prospected.

Keywords: Fluorescence lifetime imaging; skin cancer diagnosis; basal cell carcinoma; squamous cell carcinoma; malignant melanoma.

1. Introduction

In recent years, with the increase of people’s outdoor activities and the aggravation of air pollution, the incidence of skin cancer has gradually increased.1,2 Take malignant melanoma (MM) as an example, from 1975 to 2014, the incidence of MM increased year by year, as shown in Fig. 1. According to the American Cancer Society, the number of new cases of cutaneous melanoma in the United States in 2018 is estimated to be 91,270, accounting for 5.3% of all new cancer cases.3,4 In China, skin cancer accounts for about 1.5% of all malignant tumors.5 According to different types of skin cells that are primarily affected, skin cancer can
be mainly divided into basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and MM, etc.\cite{6} BCC is the most common, accounting for 65–75% of all skin tumors. SCC is prone to regional lymph node metastasis. It is much more dangerous than basal cell carcinoma. MM is a highly malignant tumor derived from neuronal melanocytes, accounting for 1% of systemic malignant tumors. It is prone to lymph node metastasis and blood spread. MM has a poor prognosis and causes the most deaths.\cite{7} As the largest organ of the integumentary system, human skin is the outer covering of the body, which provides accessibility and convenience for detection. If an appropriate method can be found for early diagnosis and timely treatment, about 90% of skin cancers can be controlled or even completely cured before they spread.\cite{8}

The traditional diagnosis of skin cancer is by biopsy and histopathological examination with hematoxylin–eosin (H&E) staining. However, it is traumatic and cannot be quantitatively detected. Recent technical advances offer many noninvasive optical detection methods, such as confocal microscopy, two-photon microscopy, Raman spectroscopy, fluorescence spectroscopy, optical coherence tomography, super-resolution microscopy imaging and multispectral imaging technique, etc.\cite{9,10,11,12,13} Among these newly developed imaging techniques, two-photon microscopy is one of the most famous landmark inventions, and fluorescence lifetime (FLT) imaging opens up new detection functions for two-photon microscopy imaging.\cite{14} Unlike intensity-based measurements, the FLT is independent of spectral similarity and concentration of multiple fluorophore labels employed and moreover it is a sensitive means for evaluating microenvironment, which makes fluorescence lifetime imaging microscopy (FLIM) a critical research tool in biomedicine by producing spatially resolved images of fluorophore lifetime.\cite{15} In recent years, the use of FLIM technology for skin cancer research has gradually increased and the statistical numbers of SCI articles since 1997 are shown in Fig. 2 (Based on the keywords of fluorescence lifetime and skin cancer, statistics in the Web of Science database).

FLIM provides a new way for cell biologists to detect, visualize, and investigate structure and function in biological systems and allows the direct diagnosis before tissue biopsy and the identification of tumor boundaries during surgery.\cite{16} Many universities and scientific research institutions in the world have performed FLIM-related researches, such as the construction on experimental platforms, FLIM data processing and analysis, biomedical diagnosis and applications.\cite{17,18,19} The company Jenlab GmbH of Germany is a pioneer in exploring the application of two-photon FLIM to clinical research and its DermaInspect system is the world’s first two-photon FLIM device for clinical skin lesion detection.\cite{12,20,21} Domestic research on FLIM started fairly late, and only a few research groups carried out this study. Among them, the group of Prof. Qu from Shenzhen University has been engaged in two-photon FLT imaging and its biomedical applications for many years and they have applied FLIM to the study on cancer identification and molecular diagnostics.\cite{22,23,24}

In this paper, the basic principle and common detection methods of FLIM will be introduced. And the latest research progresses based on FLIM in the diagnosis of three different skin cancers, including BCC, SCC and MM will be reviewed. Furthermore, the potential advantages of FLIM in skin cancer
diagnosis and the challenges that may be faced in the future will be prospected.

2. Fluorescence Lifetime Imaging Microscopy

2.1. Principle

FLT is a dynamic process of fluorescence intensity decay. When a substance is excited by a laser of a suitable wavelength, the molecule of the substance absorbs energy and transitions from the ground state to an excited state, and then emits fluorescence with longer wavelength in the form of a radiation transition to return to the ground state. The FLT refers to the average amount of time that the molecule stays in its excited state before emitting a photon. Usually, it is defined as the time required for the fluorescence intensity to decay from its peak value to 1/e of its peak value.

Specifically, after the excitation is stopped, the fluorescence intensity that is proportional to the populations in the excited state will decay exponentially with time. For a fixed fluorescent molecule, a single exponential function can be used to describe this process:

$$I(t) = I_0 e^{-t/\tau}.$$  \hfill (1)

Here, $\tau$ is the fluorescence lifetime, $t$ is the time, and $I_0$ is the initial fluorescence intensity at $t = 0$, and $I(t)$ is the fluorescence intensity at time $t$, respectively.

In biological samples, there are more than one kind of substances that generate fluorescence when excited by a laser. The superposition process of the FLT of multiple fluorescent substances can be expressed by a multi-exponential function, as shown below:

$$I(t) = \sum_{i}^{n} \alpha_i \cdot e^{-t/\tau_i}. \hfill (2)$$

where $\tau_i$ is the FLT of the $i$th fluorescent substance and $\alpha_i$ is the $i$th weighting factor.

2.2. Fluorescence lifetime measurement method

There are many methods for determining the FLT of a substance and they can be divided into two main categories: frequency domain method and time domain method.

2.2.1. Frequency domain method

The frequency domain method is also called phase-modulation method. It usually uses a sinusoidally modulated continuous-wave laser to excite a sample. The fluorescence emitted from the sample is also sinusoidally modulated with the same modulation frequency as that of the excitation source. The FLT value is calculated by measuring the demodulation coefficient and the phase shift between the fluorescence and the excitation light. Different modulation frequencies (generally the reciprocal of the fluorescence lifetime) can be chosen for different samples, thereby expanding the measurement range of fluorescence lifetime. Figure 3 shows the schematic diagram of the frequency domain method. Assuming that the phase shift of fluorescence relative to the excitation light is $\varphi$ and the modulation factor is $M$, the formulas for calculating the FLT are

$$\tau_\varphi = \frac{1}{\omega} \tan \varphi. \hfill (3)$$

$$\tau_M = \frac{1}{\omega} \sqrt{(1/M^2 - 1)}. \hfill (4)$$

$$\tau = (\tau_\varphi + \tau_M)/2. \hfill (5)$$

Here, $\omega$ is the modulation frequency; for the one-component fluorescence decay, $\tau_\varphi = \tau_M$ (where $\tau_\varphi$ is the phase lifetime and $\tau_M$ is the modulation lifetime, respectively).

2.2.2. Time domain method

The time domain method is also called the pulse method. When a sample is excited by an ultra-short optical pulse, the time-resolved fluorescence will
decay exponentially as described in Eq. (1) or (2). Measurement of FLT by time domain method mainly employs time-gated detection, streak camera and time-correlated single photon counting (TCSPC), etc.\textsuperscript{28–30} Figure 4 shows the schematic diagram of the time domain method.\textsuperscript{28} A simple case is one-component FLT measurement. By measuring the attenuation of the fluorescence intensity at different time, the FLT of a molecule is calculated with the following formula:

\[
\tau = (t_2 - t_1) / \ln\left(\frac{I_1}{I_2}\right) \text{.} \tag{6}
\]

Here, \(I_1\) and \(I_2\) are the intensities at time \(t_1\) and \(t_2\), respectively.

\section*{2.3. Fluorescence lifetime imaging microscopy system}

FLT measurement and imaging can be obtained by different implementations, either in time domain or in frequency domain as stated above. TCSPC-based FLIM has the best signal-to-noise ratio of any FLIM techniques and it is an accepted gold standard for FLT measurement. Here we show the schematic of a typical TCSPC-based FLIM system\textsuperscript{31,32} in Fig. 5.

The system mainly consists of a picosecond/femtosecond pulsed laser source, an inverted fluorescence microscope, a high speed confocal scanner, and a TCSPC module. A high repetition rate picosecond or femtosecond pulsed laser is employed to excite the sample through a laser scanning microscope. The fluorescence emitted from the sample is detected by the PMT after an emission filter and then sent to the time-correlated single photon counter for photon-number statistics. Besides the light delivered to the microscope for sample excitation, a small portion of the excitation light is used as a stop pulse for TCSPC. The time probability distribution of the arrival photons and the fluorescence intensity decay curve can be obtained. The FLT of the sample is measured point by point, and the FLT image of the region of interest is reconstructed.

\section*{3. Application of FLIM in Skin Cancer Diagnosis}

\subsection*{3.1. Basal cell carcinoma}

BCC is the most common skin cancer and occurs mainly in fair-skinned patients. It accounts for at least 32\% of all cancers globally. In the United States, about 35\% of white males and 25\% of white females are affected by BCC at some stage in their lives.\textsuperscript{33} BCC begins in the basal cell layer of the skin and can damage the tissue around it, but it has a low metastatic rate and is unlikely to cause death.

Existing research on BCC mainly focused on isolated tissues. In 2008, Galletly \textit{et al.}\textsuperscript{34} used FLIM technology to distinguish between BCC and surrounding normal skin tissue. The sample was excited by a 355 nm pulsed laser to obtain auto-FLT images of 25 unstained BCC tissues. The results showed that BCC and normal tissues could be clearly distinguished in the wide-field pseudo-color FLT image, and the FLT of BCC was shorter than that of normal skin tissue.

In 2011, based on FLIM technology, Patalay \textit{et al.}\textsuperscript{35} used two spectral channels to detect fluorescence intensity and fluorescence lifetime, and then segmented the images to calculate the FLT of the regions of interest (ROI). A 760 nm laser was
employed to excite freshly removed benign sputum (naevi) and malignant nodular basal cell carcinoma (nBCC). There was a statistically significant difference between the mean fluorescence lifetimes of nBCC and naevi, which may be derived from each spectral channel. The fluorescence lifetimes of NADH, flavin and melanin were different. In the spectral channel of < 500 nm, the FLT of NADH contributed a lot. While in the channel of > 500 nm, the FLT of melanin and flavin contributed a lot.

In 2012, Patalay et al. utilized a two-photon fluorescence imaging system with a 790 nm pulsed laser to excite the endogenous fluorophores of the isolated BCC samples and imaged the structure and morphology of the cells and extracellular matrix. By spectral scanning in the 450–700 nm band, they found that it was indistinguishable between normal epidermal tissue and BCC tissue because of their same autofluorescence emission peak at 545 nm. However, by imaging the two-photon FLT of endogenous fluorescent substances, it was found that BCC tissue has a longer FLT relative to normal skin tissue.

In 2014, Fan et al. utilized a two-photon fluorescence imaging system with a 790 nm pulsed laser to excite the endogenous fluorophores of the isolated BCC samples and imaged the structure and morphology of the cells and extracellular matrix. By spectral scanning in the 450–700 nm band, they found that it was indistinguishable between normal epidermal tissue and BCC tissue because of their same autofluorescence emission peak at 545 nm. However, by imaging the two-photon FLT of endogenous fluorescent substances, it was found that BCC tissue has a longer FLT relative to normal skin tissue.

In 2017, Luo et al. distinguished between BCC, actinic keratosis (AK) and Bowen’s disease (BD) by FLT and phasor analysis of H&E stained sections. The study found that BCC, AK and BD could be identified according to the distribution of fluorescence lifetimes of stratum corneum (SC), epidermal cells (ECs) and connective tissue (CT), as shown in Fig. 6. In addition, the analysis of the FLT of H&E stained tissue in the phasor space was performed by observing the phasor diagram. The coordinate values, diagonal slopes, and phasor regions of the skin layers in the vector diagram were different. FLT imaging microscopy and phasor method (phasor-FLIM) provided a simple, noninvasive histopathological analysis for the diagnosis of skin cancer.

### 3.2. Squamous cell carcinoma

SCC, the second most common form of skin cancer is also known as epidermal carcinoma. More than 1 million cases of SCC are diagnosed and more than 15,000 people die from the disease each year in the United States. SCC is a malignant tumor that mostly occurs in the squamous epithelium and usually results from human papillomavirus heat damage, ultra violet radiation and chemical carcinogens, etc. SCC is clinically and histopathologically diverse and the cancer cells have different degrees of keratinization at different stages of development. Early diagnosis and treatment can inhibit cancer cells to a certain extent.

In 2010, Martin-Villar et al. used in vivo fluorescence resonance energy transfer/fluorescence lifetime imaging microscopy (FRET/FLIM) to measure the interactions between Podoplanin-eGFP (donor) and CD44s-mRFP (receptor). The experimental results showed that the expression of Podoplanin in tumor cells, especially SCCs, was linked to increased cell migration and invasiveness, and Podoplanin-CD44s interaction was associated on the plasma membrane of cells with a migratory phenotype. FRET/FLIM demonstrated that the Podoplanin-CD44s interaction was important for driving tumor cell migration during malignancy.

In 2011, Roberts et al. used FLIM to study skin conditions. The FLIM images of the stratum granulosum, stratum spinosum and stratum basale from healthy, psoriasis lesion, atopic dermatitis lesion and SCC lesion skin were shown respectively. According to FLT decay of double-exponential function, the ratio of amplitudes \( \frac{a_1}{a_2} \) of NAD(P)-H was used as an indication of metabolic rate. The results showed that the metabolic rates differed depending on the skin conditions. Although FLIM could distinguish between different lesions and healthy skin, its combination with multiple complementary modalities such as spectral imaging, reflectance and Raman imaging with confocal and...
multiphoton spectroscopy were suggested to achieve robust diagnostic discrimination between skin diseases and their boundaries.

In 2015, Miller et al. used intensity, time and wavelength resolved multiphoton microscopy to measure changes in cellular metabolism. By analyzing the fluorescence emission of metabolic enzymes such as the reduced form of NADH and the oxidized form of flavin cofactors, slight changes in the mitochondrial microenvironment and cellular metabolism can be quantified. Dual-channel FLIM was used to quantify metabolic changes both within and between high- and low-HER2 expressing SCC cell lines (SCC-74B and SCC-74A, respectively). Chemically induced changes in metabolic state resulted in measurable changes in lifetime distribution and fluorescence intensity within each cell line. In addition, SCC-74A and SCC-74B could be clearly distinguished based on the dynamic range of fluorescence intensity (the difference between uncoupled and inhibited metabolic states) and the lifetime distribution.

In 2017, Miller et al. characterized skin cancer in living mice with SCC by using multimodal fluorescence molecular imaging method. Firstly, the endogenous fluorophores, such as flavin and lipofuscin, were excited by 480 nm laser. According to the auto-FLT imaging, it was found that the short-lived component in SCC increased relative to normal skin, indicating that the FLT of the tumor was shorter than that of normal skin, as shown in Fig. 7. In addition, a near-infrared (NIR) fluorescent molecular probe was injected into a mouse suffering from SCC and subjected to fluorescence molecular tomography (FMT), which provided a method to estimate the tumor volume and depth, as well as quantify the molecular probe concentration in SCC. All detection and imaging methods in this study were performed on live mice, providing a comprehensive model for skin tumor research.
3.3. **Malignant melanoma**

MM is a very aggressive and dangerous type of cancer that develops in the pigment-containing cells known as melanocytes. An estimated 178,560 cases of melanoma were diagnosed in the United States in 2018. It is especially important for MM’s early diagnosis and treatment because of its high malignancy, easy to early metastasis and high mortality. Early stage melanoma has an overall survival rate of nearly 100%, while metastatic melanoma can be rapidly fatal. Until now, MM has been studied using cell models, *ex vivo* tissues and animal models.

In 2008, Cicchi *et al.* used the two-photon FLIM experimental system to perform lifetime imaging analysis of MM and melanocytic nevus (MN). They evaluated the lifetime distribution of a layer containing MM or MN cells using both single- and double-exponential decay function. The results showed that MM exhibited a broader mean lifetime distribution with respect to the corresponding MN mean lifetime distribution in both single-exponential and double-exponential decay analysis, and the differentiation between MM and MN occurred at the fast lifetime component level in double-decay analysis. Therefore, it could be discriminated between MM and MN by looking at the different
behaviors in the lifetime domain of the acquired image.

In 2009, by selective melanin imaging and spectral FLT imaging, Dimitrow et al.\textsuperscript{45} found that the FLT distribution was in correlation with the intracellular amount of melanin. Compared to keratinocytes, MM exhibited a greater ratio of the intensity coefficients $a_1/a_2$ ($a_1$ was the shorter component and $a_2$ was the longer component), so MM had a shorter fluorescence lifetime. Procedures of selective imaging as well as spectral FLT imaging supported diagnostic decisions and could improve the process of noninvasive early detection of melanoma.

In 2014, Pires et al.\textsuperscript{46} performed experiments on 42 experimental melanoma lesions induced in balb/c nude mice using the cell line B16F10, with a 378 nm laser to excite NADH and a 445 nm laser to excite FAD. The experimental results showed that the two methods could distinguish normal skin and melanoma according to the change of fluorescence lifetime. In practice, the contribution of various endogenous fluorescent molecules in the tissue should be considered comprehensively. Using this time-domain fluorescence spectroscopy to detect melanoma in animal models, the sensitivity of 99.4%, specificity of 97.4% and accuracy of 98.4% were achieved.

In 2017, Pastore et al.\textsuperscript{47} used a melanoma mouse model to compare the morphological and metabolic state changes of normal skin and melanoma tissue at three different growth stages based on NADH.

![Fig. 8. Multiphoton FLIM on freshly excised mouse ear skin for different stages of melanoma development. (a) Representative FLIM images of the stratum granulosum/spinosum (left column) and stratum basale (right column) layers, pseudocolored from 2 (blue) to 5 (red) according to the ratio of the amplitudes $a_1$ and $a_2$, reflecting the portion of free and bound NADH. White numbers indicate cells selected for further analysis. (b) Boxplots of the ratios of the amplitudes $a_1/a_2$ of the marked cells ((a), white numbers within FLIM images); centre line indicates the median; symbols: value significantly different compared to (*) control, (#) < 0.5 mm lesion size and (†) ~1.5 mm lesion size. Statistical analysis performed using Kruskal–Wallis test followed post hoc with Dunn’s multiple comparison test. Significance was $p < 0.05$.\textsuperscript{47}](image)
signaling. It was found that with the growth of melanoma, the ratio of free NADH to protein-bound NADH increased significantly (Fig. 8), while the mean FLT of NADH decreased. This study demonstrated that the use of two-photon FLIM to detect the metabolic state characterized by NADH could quickly and sensitively reflect the growth stage of melanoma. FLIM technology had the potential to be a powerful tool for the diagnosis and staging of melanoma.

4. Summary

Skin cancer is the most common form of cancer that is caused mostly by exposure to ultraviolet radiation from the sun. Research shows that skin cancer is associated with abnormal cellular metabolism and its early identification may introduce the possibility of intervention to prevent its progress to a deadly metastatic stage. FLIM enables noninvasive detection of complex molecular assemblies for studies of cell function and dynamics with subcellular resolution by imaging the lifetime of the fluorophore signal rather than its intensity. It can be used as an imaging technique in confocal microscopy, two-photon excitation microscopy and multiphoton tomography, etc. With these advantages, FLIM has been performed as a powerful imaging tool in cell biology and biomedicine.

This paper briefly summarizes the principle and detection methods of FLIM and reviews in detail its applications in the diagnosis of skin cancer, including BCC, SCC and MM. By measuring the FLT of endogenous (such as NADH, FAD, etc.) or exogenous fluorophores, FLIM provides information on both morphology and metabolism of the tissues under investigation at a subcellular level, which helps differentiate between normal skin tissue and cancerous tissue as well as presents a great potential in clinical application.

However, the application of FLIM in skin cancer is still in its infancy. No strict definition to quantitatively describe normal skin tissue and cancerous tissue, limitations in hardware systems and imaging principles, and lack of proper fluorescent indicators, are the great challenges that FLIM faces in the study of skin cancer. The FLIM system used in clinical dermatology should be noninvasive, low cost, compact and portable, and with high speed, sensitivity and accuracy. Combination with multiple techniques, such as dermoscopy, will help to improve the system performance and efficiency. We believe that with the development of imaging and detection technologies and the improvement of data analysis capabilities, FLIM, as a noninvasive pathological research tool, will play a role in the early diagnosis and treatment of skin cancer.

Conflict of Interest

There are no conflicts to declare.

Acknowledgments

This work has been partially supported by The 111 Project (B17035), Open Research Fund Program of the State Key Laboratory of Low Dimensional Quantum Physics (KF201713), State Key Laboratory of Transient Optics and Photonics, Chinese Academy of Sciences (SKLST201804) and the Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province (GD201711).

References

1. Z. Apalla, A. Lallas, E. Sotiriou, L. Elizabeth, I. Demetrios, “Epidemiological trends in skin cancer,” Dermatol. Pract. Concept. 7(2), 1–6 (2017).
2. Z. Apalla, D. Nashan, R. B. Weller, X. Castellsagué, “Skin cancer: Epidemiology, disease burden, pathophysiology, diagnosis, and therapeutic approaches,” Dermatol. Ther. 7(1), 5–19 (2017).
3. American Cancer Society, Cancer Facts & Figures 2018, https://www.cancer.org/research/cancer-facts-figures/all-cancer-facts-figures/cancer-facts-figures-2018.html (2018).
4. National Cancer Institute, SEER stats fact sheets: melanoma of the skin, https://seer.cancer.gov/statfacts/html/melan.html (2018).
5. J. Yang, S. Xiong, “Diagnosis and treatment of human basal cell carcinoma and squamous cell carcinoma,” China Mod. Med. 18(11), 13–15 (2011).
6. A. Luu, L. Doerwald-Munoz, O. Stapiak, “An evaluation of two approaches to skin bolus design for patients receiving radiotherapy for head and neck cancers,” J. Med. Imaging Radiat. Sci. 46(3), 37–42 (2014).
7. Z. Tang, J. Shi, M. Cai, D. Xue, “An analysis of skin melanoma incidence and its influencing factors in China,” China Cancer 23(10), 829–833 (2014).
8. Y. Lu, X. Peng, S. Fan, S. Liu, J. Qu, “Application of new optical methods in skin cancer research,” Int. J. Dermatol. Venereol. 40(6), 383–385 (2014).
9. M. Y. Berezin, S. Achilefu, “Fluorescence lifetime measurements and biological imaging,” Chem. Rev. 110(5), 2641–2684 (2010).
10. V. Ntiachristos, “Going deeper than microscopy: The optical imaging frontier in biology,” Nat. Methods 7(8), 603–614 (2010).
11. K. Nienhaus, G. U. Nienhaus, “Where do we stand with super-resolution optical microscopy,” J. Mol. Biol. 428(2), 302–322 (2016).
12. K. König, “Clinical multiphoton tomography,” J. Biophoton. 1(1), 13–23 (2008).
13. S. G. Stanciu, M. Costache, D. E. Tranca, R. Hristu, M. Popescu, V. Enache, G. A. Stanciu, “Towards imaging skin cancer by apertureless scanning near-field optical microscopy,” U.P.B. Sci. Bull., Ser. A. Appl. Math. Phys. 78(2), 235–244 (2016).
14. X. Zhang, “Imaging technology in modern microsystems,” Opt. Instrum. 37(6), 550–560 (2015).
15. V. V. Ghukasyan, F. J. Kao, “Monitoring cellular metabolism with fluorescence lifetime of reduced nicotinamide adenine dinucleotide,” J. Phys. Chem. 113(27), 11532–11540 (2009).
16. M. W. Conklin, P. P. Provenzano, K. W. Eliceiri, R. Sullivan, P. J. Keely, “Fluorescence lifetime imaging of endogenous fluorophores in histopathology sections reveals differences between normal and tumor epithelium in carcinoma in situ of the breast,” Cell Biochem. Biophys. 53(3), 145–157 (2009).
17. L. Marcu, “Fluorescence lifetime techniques in medical applications,” Ann. Biomed. Eng. 40(2), 304–331 (2012).
18. D. Chorvat, A. Chorvatova, “Multi-wavelength fluorescence lifetime spectroscopy: A new approach to the study of endogenous fluorescence in living cells and tissues,” Laser Phys. Lett. 6(3), 175–193 (2009).
19. M. S. Roberts, Y. Dancik, T. W. Prow, C. A. Thorling, L. L. Lin, J. E. Grice, T. A. Robertson, K. König, W. Becker, “Non-invasive imaging of skin physiology and percutaneous penetration using fluorescence spectral and lifetime imaging with multiphoton and confocal microscopy,” Eur. J. Pharm. Biopharm. 77(3), 469–488 (2011).
20. J. Leppert, J. Krajewski, S. R. Kantelhardt, S. M. Schäffler, N. Petkus, E. Reusche, G. Hüttmann, A. Giese, “Multiphoton excitation of autofluorescence for microscopy of glioma tissue,” Neurosurgery 58(4), 759–767 (2006).
21. I. Riemann, A. Ehlers, R. Le Harzic, E. Dimitrow, M. Kaatz, P. Elsner, R. Bückle, K. Koenig, “Non-invasive analysis/diagnosis of human normal and melanoma skin tissues with two-photon FLIM in vivo,” Proc. SPIE 6842, 684205 (2008).
22. S. Fan, X. Peng, L. Liu, S. Liu, Y. Lu, J. Qu, “Diagnosis of basal cell carcinoma by two photon excited fluorescence combined with lifetime imaging,” Proc. SPIE 8948, 89482E (2014).
23. A. Pliss, X. Peng, L. Liu, A. Kuzmin, Y. Wang, J. Qu, Y. Li, P. N. Prasad, “Single cell assay for molecular diagnostics and medicine: Monitoring intracellular concentrations of macromolecules by two-photon fluorescence lifetime imaging,” Theranostics 5(9), 919–930 (2015).
24. A. Pliss, S. M. Levchenko, L. Liu, X. Peng, T. Y. Ohulchanskyy, I. Roy, A. N. Kuzmin, J. Qu, P. N. Prasad, “Cycles of protein condensation and discharge in nuclear organelles studied by fluorescence lifetime imaging,” Nat. Commun. 10(1), 455 (2019).
25. TuanVo-Dinh, Biomedical Photonics Handbook, CRC Publishing Company, Florida (2003).
26. A. T. N. Kumar, S. B. Raymond, B. J. Bacskaï, D. A. Boas, “Comparison of frequency-domain and time-domain fluorescence lifetime tomography,” Opt. Lett. 33(5), 470–472 (2008).
27. E. Gratton, S. Breusegem, J. Sutin, Q. Q. Ruan, N. Barry, “Fluorescence lifetime imaging of two-photon microscopy: Time domain and frequency domain method,” J. Biomed. Opt. 8(3), 381–390 (2003).
28. L. Liu, J. Qu, Z. Lin, B. Guo, H. Niu, “Fluorescence lifetime imaging and its biomedical applications,” J. Shenzhen Univ. Sci. Eng. 22(2), 133–141 (2005).
29. W. Becker, A. Bergmann, K. Koenig, U. Tirlapur, “Picosecond fluorescence lifetime microscopy by TCSPC imaging,” Proc. SPIE 4262, 414–419 (2001).
30. X. Liu, D. Lin, Q. Wu, W. Yan, T. Luo, Z. Yang, J. Qu, “Recent progress of fluorescence lifetime imaging microscopy technology and its application,” Acta Phys. Sin. 67(17), 27–40 (2018).
31. K. Suhling, L. M. Hirvonen, J. A. Levitt, P. H. Chung, C. Tregido, A. Le Marois, D. Rusakov, K. Zheng, S. Anmer-Beg, S. Poland, S. Coelho, R. Dimble, “Fluorescence lifetime imaging (FLIM): Basic concepts and recent applications,” Springer Ser. Chem. Phys. 111, 119–188 (2015).
32. L. Liu, A. Pliss, X. Peng, A. Kuzmin, J. Qu, P. N. Prasa, “Mapping of intracellular concentrations of macromolecules by two-photon excited fluorescence lifetime imaging,” Proc. SPIE 9712, 971221 (2016).
33. S. A. Gandhi, J. Kampp, “Skin cancer epidemiology, detection, and management,” Med. Clin. N. Am. 99(6), 1323–1335 (2015).
34. N. P. Galletly, J. Mccinty, C. Dunsby, F. Teixeira, J. Requejo-Isidro, I. Muro, D. S. Elson, M. A. Neil, A. C. Chu, P. M. French, G. W. Stamp, “Fluorescence lifetime imaging distinguishes basal cell
carcinoma from surrounding uninvolved skin,” Br. J. Dermatol. **159**(1), 152–161 (2008).
35. R. Patalay, C. B. Talbot, L. Munro, H. G. Breunig, “Fluorescence lifetime imaging of skin cancer,” *Proc. SPIE* **7883**, 78830A (2011).
36. R. Patalay, C. Talbot, Y. Alexandrov, I. Munro, M. A. Neil, K. König, P. M. French, A. Chu, G. W. Stamp, C. Dunsby, “Quantification of cellular autofluorescence of human skin using multiphoton tomography and fluorescence lifetime imaging in two spectral detection channels,” *Biomed. Opt. Exp.* **2**(12), 3295–3308 (2012).
37. S. Seidenari, F. Arginelli, C. Dunsby, P. French, K. Koenig, C. Magnoni, M. Manfredini, C. Talbot, G. Ponti, “Multiphoton laser tomography and fluorescence lifetime imaging of basal cell carcinoma: Morphologic features for non-invasive diagnostics,” *Exp. Dermatol.* **21**(11), 831–836 (2012).
38. T. Luo, Y. Lu, S. Liu, D. Lin, J. Qu, “Phasor-FLIM as a screening tool for the differential diagnosis of actinic keratosis, Bowen’s disease and basal cell carcinoma,” *Anal. Chem.* **89**(15), 8104–8111 (2017).
39. T. Zhao, J. Zheng, “Advances in the research of cutaneous squamous cell carcinoma,” *Int. J. Dermatol.* **32**(4), 247–250 (2006).
40. E. Martín-Villar, B. Fernández-Muñoz, M. Parsons, M. M. Yurrita, D. Megías, E. Pérez-Gómez, G. E. Jones, M. Quintanilla, “Podoplanin associates with cd44 to promote directional cell migration,” *Mol. Biol. Cell* **21**(24), 4387–4399 (2010).
41. C. R. Miller, M. G. Nichols, “Metabolic profiling of the skin to monitor the onset and progression of squamous cell carcinoma through time- and wavelength-resolved fluorescence lifetime imaging,” *Biophys. J.* **108**(2), 478a (2015).
42. J. P. Miller, L. M. Habimana-Griffin, T. S. Edwards, S. Achilefu, “Multimodal fluorescence molecular imaging for in vivo characterization of skin cancer using endogenous and exogenous fluorophores,” *J. Biomed. Opt.* **22**(6), 066007 (2017).
43. S. LévéqueFort, M. P. Fontaineaupart, G. Roger, P. Georges, “Fluorescence-lifetime imaging with a multifocal two-photon microscope,” *Opt. Lett.* **29**(24), 2884–2886 (2004).
44. R. Cicchi, S. Sestini, V. D. Giorgi, D. Massi, T. Lotti, F. S. Pavone, “Multidimensional two-photon imaging of diseased skin,” *Proc. SPIE* **6859**, 685903 (2008).
45. E. Dimitrow, I. Riemann, A. Ehlers, M. J. Koehler, J. Norgauer, P. Elsner, K. Koenig, M. Kaatz, “Spectral fluorescence lifetime detection and selective melanin imaging by multiphoton laser tomography for melanoma diagnosis,” *Exp. Dermatol.* **18**(6), 509–515 (2009).
46. L. Pires, M. S. Nogueira, S. Pratavieira, L. Moriyama, C. Kurachi, “Time-resolved fluorescence lifetime for cutaneous melanoma detection,” *Biomed. Opt. Express* **5**(9), 3080–3089 (2014).
47. M. N. Pastore, H. Studier, C. S. Bonder, M. Roberts, “Non-invasive metabolic imaging of melanoma progression,” *Exp. Dermatol.* **26**(7), 607–614 (2017).