Recent trends in two-photon auto-fluorescence lifetime imaging (2P-FLIM) and its biomedical applications

Harsh Ranawat¹ · Sagnik Pal¹ · Nirmal Mazumder¹

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Abstract

Two photon fluorescence microscopy and the numerous technical advances to it have served as valuable tools in biomedical research. The fluorophores (exogenous or endogenous) absorb light and emit lower energy photons than the absorption energy and the emission (fluorescence) signal is measured using a fluorescence decay graph. Additionally, high spatial resolution images can be acquired in two photon fluorescence lifetime imaging (2P-FLIM) with improved penetration depth which helps in detection of fluorescence signal in vivo. 2P-FLIM is a non-invasive imaging technique in order to visualize cellular metabolic, by tracking intrinsic fluorophores present in it, such as nicotinamide adenine dinucleotide, flavin adenine dinucleotide and tryptophan etc. 2P-FLIM of these molecules enable the visualization of metabolic alterations, non-invasively. This comprehensive review discusses the numerous applications of 2P-FLIM towards cancer, neuro-degenerative, infectious diseases, and wound healing.

Keywords Two-photon excitation · Fluorescence lifetime imaging microscopy (FLIM) · Nicotinamide adenine dinucleotide hydrogen (NADH) · Flavin adenine dinucleotide (FAD)

1 Introduction

Fluorescence emission based techniques using dyes for optical microscopy, imaging and spectroscopy have been used extensively in the recent years, though their potential was initially underestimated. However, metabolic properties of cells cannot be well understood by this technique. Furthermore, the ambiguous background signals due to cellular auto-fluorescence are a major shortcoming in fluorescence microscopy [1–3]. To acquire three-dimensional (3D) images of tissues with sufficient depth penetration, optical tissue clearing and immunofluorescence techniques have to be employed [1]. This results in masking of the original signal from the biomolecules in cells. Hence, more advanced fluorescence based microscopy and spectroscopy

are needed. Over the past decade, there has been an increase in the use of fluorescence lifetime imaging (FLIM) techniques in biomedical research [4, 5]. Fluorescence lifetimes serve as important parameter in studying the effect of chemical factors such as pH, ions and molecularity in biological systems [6–9]. Optical microscopy based on fluorescence detection has a wide range of applications in medical biotechnology, diagnosis, DNA sequencing, flow cytometry and forensics. Spatial organization and quantification of protein–protein interactions is enabled using Forster resonance energy transfer (FRET) and FLIM, both in vivo and in vitro [10–14]. FLIM is frequently employed in the study of intracellular energy transfer due to molecular interactions. The signals are a function of intermolecular distance, and hence enable the evaluation of molecular gaps at a better resolution when compared to conventional optical imaging techniques [2, 15, 16]. A ground state fluorophore is excited to a higher energy state by absorption of photons of particular energy, corresponding to the energy difference between electronic states. This can occur by single or multi-photon absorptions in a single quantum event. The excited electron of the fluorophore returns to the ground state through a number of radiative and non-radiative processes, the probability of which is taken into account for the decay rates. The lifetime

Harsh Ranawat and Sagnik Pal have contributed equally to this work.

✉ Nirmal Mazumder
nirmaluva@gmail.com

¹ Department of Biophysics, Manipal School of Life Sciences, Manipal Academy of Higher Education, Manipal, Karnataka 576104, India

can therefore be defined as the average time a fluorophore spends in the excited state before returning to the ground state [1, 17]. This lifetime is affected by the physical and chemical surroundings of the fluorophore is in, and is a fundamental parameter, which makes it independent of its concentration in the cells. It is also independent of the optical path of the microscope or photo-bleaching effects, and this property is exploited to structure probes and study their dynamics at the micro and nano-scales. A single exponential decay function is applicable in case of homogeneous ensemble of fluorescence decays with time. Hence, for specific fluorophores these lifetimes can serve as markers, but in case of multi-exponential decays, deciphering linear correlations between structural conformations and decay components is tedious [2, 5].

The advantage of intrinsic fluorescence over extrinsic fluorescence lies in the undisturbed environment during measurement. Auto-fluorescence spectroscopy is used in diagnostic studies such as Alzheimer disease, atherosclerosis and others—where the difference in concentration of fluorophores and changes in their environment cause a change in lifetime, forming the basis of identification [18–20]. In contrast to bulk auto-fluorescence spectra, the fluorescence lifetimes serve as a fingerprint, providing contrast between different molecules and/or different molecule's configurations. The fluorescence lifetime of a fluorophore is detected under single photon as well as multiphoton microscope setups by time correlated single photon counting (TCSPC)/phasor analysis [2, 5]. NAD, the primary electron acceptor, is fluorescent in its reduced form-NADH [8, 10]. When NAD is bound to dehydrogenase enzymes, a blue shift in both excitation and emission maxima are observed, along with an increase in quantum yield of fluorescence. Again, FAD is fluorescent in its oxidized state. Protein-bound NADH and free FAD molecules show long lifetime components, while free NADH and protein bound FAD molecules exhibit shorter lifetime components. The shorter fluorescence lifetime components of both free NADH and protein-bound FAD are due to the dynamic quenching of adenine moiety [22–24]. Fluorescence lifetime measurements allow differentiation of free and protein-bound components of NADH and FAD while also calculating relative amounts of these species [21]. This ability was applied in categorizing epithelial cells as normal or cancerous and also to discriminate benign and aggressive tumor cells [14]. Characterization of the fluorescence lifetime of NADH has been carried out in a variety of biological samples, including pathological conditions, in isolated mitochondria [23, 24], in vitro cell cultures [10, 19, 25, 26], ex vivo [27–29] and in vivo measurements [30–33]. Tryptophan (Trp) is an aromatic amino acid, crucial in determining protein composition, structure and micro-environment, hence can be used as a marker for protein abundance. It has also been linked to various oncological investigations

[12, 34, 35]. In an earlier study focusing on FLIM of tryptophan in lysozymes revealed quenching mechanisms between Trp-62 and Trp-108. Lifetime of Trp-108 was reduced in the presence of Trp-62, whose photon emission counts had increased. Also, the steady state fluorescence intensity of lysozyme on binding with N-Acetyl Glucosamine (NAG) decreases at pH 2 and 5, unlike at pH 8 [34]. Long excitation wavelength of 590 nm was used to image leukocytes by their intrinsic tryptophan fluorescence by two photon absorption. This non-invasive method allowed for optical sectioning through the epidermal to dermal cell layers of mouse skin, and even at recording time of 30 min, no significant photo-bleaching was experienced by the skin. This study demonstrated the potential of 2P-FLIM measurements in tracking cellular movement and monitoring inflammatory cellular reactions [35]. In another study, three photon fluorescence spectroscopy of tryptophan revealed a decrease in lifetime from 3.02 to 1.54 ns in the presence of NADH. This quenching effect was more noticeable in tumorigenic HeLa cells, attributed to increased protein-bound NADH due to elevated glycolysis rate and subsequent oxidative phosphorylation, relative to non-tumorigenic MCF10A cells [12].

2P-FLIM meets the needs of neuroscientific research because of the critical advantages—superior depth penetration and minimum photo-bleaching owing to long excitation wavelengths and inherent optical sectioning unlike confocal microscopy [36, 37]. Since absorption rate is steeply dependent on photon concentration, the emitted fluorescence is largely confined to the focal point of excitation laser—causing an inherent optical sectioning. A large variety of excitation wavelengths and emitted colors are available in synthetic dyes and fluorescent proteins [38, 39]. Intravascular injection of fluorescent dextran enables study of blood flow dynamics at the capillary level in superficial cortex layers. Methoxy X-04 was used as blood-brain barrier amyloid-beta marker in course models of Alzheimer's disease [40, 41]. In calcium imaging experiments, Sulforhodamine 101 has been used as a contrast agent as it binds to the glia [42]. Nanoparticles have proved to be a significant tool at the disposal of biomedical research, however, methods to detect and determine required action still remain relatively unimproved. Recent studies using FLIM allow for the determination of these nanoparticles at various stages of the application: (a) To study the kinetics of formation of organic nanoparticles: progression through the monomeric, cluster and nanoparticle stages in a three-dimensional hydrodynamic focusing microfluidics method was indicated by 10 fold decrease in fluorescence lifetime [43]; (b) Visualization of uptake and intracellular localization: confocal microscopy for fluorescence intensity and fluorescence lifetime of quantum dots enabled determination of the endocytic pathway in fibroblasts. Endosomes at different stages of

maturity also exhibited different lifetimes [44]; (c) Release of drugs: Intracellular release of doxorubicin was monitored by the difference in lifetime between native DOX (~1 ns) and IONP-conjugated DOX (~4.6 ns), estimated using phasor plots [45].

Retinal in vivo fluorescence imaging is one of the exclusive methods for the investigation of the structure as well as the function of the retina in both healthy and disease case. Consolidation of the contrast supplemented by fluorescence techniques and adaptive optics have made the determination of morphological characteristics of cell types in the retina using single [46–49] and multi-photon excitation techniques [50–52]. A recent study demonstrated the functional ability of Adaptive Optics Two Photon Excited Fluorescence Lifetime Imaging Ophthalmoscopy (AOFLIO) in imaging the extrinsic fluorophores in mouse retina. The multiple functional responses are obtained by measuring the changes in the fluorescence signal in in vivo using genetically encoded calcium indicators and two photon auto-fluorescence in both murine and mouse models. This study also opened doors for future application of molecular targeting and study of disease progression [53]. Disease progression can be detected in an earlier stage of patients with diabetic retinopathy and Stargardt disease through the changes observed in the fluorescence lifetime imaging of the retina in vivo. Till now, retinal in vivo fluorescence lifetime imaging has been only subjected to canonical resolution instruments which make it quite challenging to identify and differentiate fluorophores in different retinal layers.

FLIM promises to provide greater biochemical and medical diagnostic information than conventional fluorescence intensity based methods for identifying abnormal tissues in vivo. Signs of psoriasis, an autoimmune inflammatory disease, could be identified in fibroblasts cells owing to increased lifetime of NADH along with changed morphology [54]. Accurate determination of mitochondria in inflammation was made possible, non-invasively, by multiphoton tomography combined with FLIM (MPT-FLIM) [55]. FLIM focused on calcium enables study of neural circuits and local brain function [56]. It has also been used in measurement of cells density in tumor cells at the cellular and sub cellular levels of glioma tissue, in a label-free approach [56]. FLIM is also able to distinguish between collagenous and lipid-rich regions of carotid plaques, which is an important parameter in checking the integrity of fibrous caps. It was also used assess the extent of plaque rupture by collagen auto-fluorescence alone, without the need of contrast agents [57]. Hence, 2P-FLIM is a constantly developing tool with increasing areas of applications. In this review, we attempt to surface the advancements in the use of 2P-FLIM for biological research. Significant areas of study such as early detection of diseases, determination of wound healing, neuroscientific and oncological research have been discussed in detail.

2 Two photon excitation and data analysis

Fluorescence lifetime of a molecule is measured using both time domain (TD) as well as frequency domain (FD) in single photon and two photon microscopy [1, 58]. Advantages of FLIM on a multiphoton laser scanning microscope is especially interesting as it allows deep imaging capability. Additionally, FLIM set-ups predominantly have the ability to conduct FRET experiments through reduction in donor's fluorescence lifetime. Since FLIM is independent of emission intensity, it serves as an essential technique for attaining information regarding the FRET tools uses in knowing the interactions of the molecules. Excited light with short pulses are used for time domain measurements and TCSPC or gated detection systems are used for directly evaluating the fluorescence decay. Modulated periodic intensity of excitation source are used for evaluating frequency-domain FLIM and identification through phase sensitive pulsed lasers with high repetitive rate or waves that are continuous and can modulate rapidly need to be used for both time as well as frequency domain FLIM systems and they are the most expensive components of the set-up [28].

A typical two photon fluorescence (2PF) TCSPC instrumentation (Fig. 1a) can be found in earlier publications [15, 16]. TD measurements employ pulsed lasers, of the width smaller than decay time (τ) to excite the fluorophores. Intensity of emitted light is recorded as a function of time. τ is determined by the slope of the counts against time graph, called the decay curve (Fig. 1b). TCSPC employs a high repetition rate pulsed laser and single photons are measured. TD measurements in imaging involve laser scanning microscopes, generally in conjunction with pico or femtosecond lasers and detectors of comparable temporal resolutions; photo-multiplier tubes (PMT) and special single photon detecting electronics. Calculation of lifetime from multi-exponential decay functions is carried out by convoluting the model function and instrument response function (IRF), along with fitting procedures. Lifetimes from composite decays of biomolecules are usually resolved using the double-exponential function and IRF [1, 2]. Figure 1 c and d represent color-coded fluorescence lifetime image of NAD(P)H in Huh 7 cells and NAD(P)H fluorescence intensity.

3 Two photon fluorescence lifetime microscopy in infectious disease

Hepatitis C Virus (HCV) infection is the leading cause of hepatocellular carcinoma and liver transplantation which is a global health concern [59]. Hepatic lipids are

Fig. 1 **a** Typical 2P-FLIM experimental set up using TCSPC: t —pulse interval, EM—emission filter, RB—reference beam, BS—beam splitter, TCSPC—time correlated single photon counter, PD—photodiode, PMT—photo multiplier tube. **b** NAD(P)H fluorescence decay curve depicted by a typical bi-exponential model with χ^2 close to 1

and no visible systematic variations shown by the residuals. **c** Representative color-coded image of NAD(P)H lifetimes in Huh7 cells and **d**. Fluorescence intensity of NAD(P)H. Scale bar = 10 μ m. Figure reproduced (from Ref. [15]) with kind permission from Springer

closely linked with the pathogenesis of HCV, with steatosis observed in over 40% patients. Intercellular lipid droplets (LDs), essential for the replication process of HCV, are formed by increased lipid synthesis and catabolism [60]. The underlying process of metabolism is not yet fully understood. The HCV core protein is associated strongly with LDs in the progression of the infection, and independently induces towards cellular lipid catabolism. D2, one of the two domains in HCV core protein, can independently change the bound protein composition, localization and trafficking of cytoplasmic LDs [61].

Mazumder et al. applied two photon fluorescence microscopy integrated to coherent anti-Stokes Raman scattering (CARS) microscopy and FLIM to investigate the metabolic signatures of NAD(P)H, presence/absence of HCV core D2 protein and LD size alterations in Huh7 cells [20]. In CARS, multiple photons are used to excite the electrons to a vibrational Eigen state and then emit photons in the anti-Stokes region i.e. able shifted in comparison to the excitation light. This eliminates background fluorescence from the sample and the CARS intensity per molecule is greater than that of conventional Raman spectroscopy, since CARS is a higher order non-linear process [62]. Huh7 cells were infected with vector expressing D2-GFP fusion protein (Fig. 2a).

2PF and CARS simultaneously revealed increase in the number and size of LDs, the D2-GFP protein being localized around them. Also, quantitative RT-PCR analysis revealed 30% decrease in PPAR- α (transcription factor involved in fatty acid catabolism)—expression. 2PF excitation at 740 nm coupled with an emission filter of 450/40 nm allowed for detection of NAD(P)H auto-fluorescence, devoid of signals from GFP. Transfected cells showed a threefold increase in auto-fluorescence intensity from NAD(P)H. Moreover, the signal was diffused throughout the cell, unlike in control where signals were localized to distant foci (mostly mitochondria)—as calculated by coefficient of variance analysis of the auto-fluorescence signals (Fig. 2b) [26]. 2P-FLIM analysis of NAD(P)H in transfected cells exhibited significant decrease in the lifetimes of free (τ_1) and bound (τ_2) form (Fig. 2c, d). τ_1 decreased from 0.49 ± 0.9 to 0.31 ± 0.3 ns while τ_2 decreased from 3.0 ± 0.3 to 2.4 ± 0.2 ns, such that the average lifetime (τ_{avg}) decreased from 2.3 ± 0.3 to 1.5 ± 0.3 ns. Analysis of amplitudes using the bi-exponential model of 2P-FLIM measurement allowed for calculation of free to protein-bound NAD(P)H (a_1/a_2). This ratio increased threefold in D2-GFP infected cells, indicating higher population of shorter-lifetime moieties. After further analysis, an estimate of 10-fold increase in NAD(P)H could be made—largely contributed by free NAD(P)H

Fig. 2 HCV-D2 core protein induced changes in NADH lifetime. Huh7 cells were transfected to express GFP and imaged for NAD(P)H lifetimes, DIC, and CCD camera, after 24 h of transfection with FLIM **a** Left panels: Average NAD(P)H lifetimes illustrated by pseudo-colored images. Right panels: DIC and GFP fluorescence (red) images overlaid. **b** Mean NAD(P)H lifetimes after quantitative

analysis. **c, d** Quantitative analysis of average lifetimes between GFP or D2-GFP expressing cells and non-transfected neighbouring cells for free (**c**) and bound (**d**) NAD(P)H. Error bars represent standard deviation. Figure reproduced (from Ref. [20]) with kind permission from PLoS One

in solution. In conclusion, FLIM enabled an independent assessment and quantification of molecular level changes in NAD(P)H. A model explaining metabolic changes caused by D2 protein of HCV has been proposed, which involves a number of mechanisms. Rapid influx of Ca^{2+} ions in the mitochondria can cause generation of reactive oxygen species (ROS) and accumulation of oxidized glutathione (GSSG) causing disruptions the electron transport chain. Alternatively, the D2 protein may directly inhibit the activity of complex I, causing an increasing in the cellular NADH concentration. This increase in NAD(P)H allows for anabolism of lipids within the infected cells.

NADH functions as a co-enzyme in the process of oxidative phosphorylation (aerobic respiration) as well as in glycolysis (anaerobic respiration). The bound form of NADH is generally in conjunction with the complex I dehydrogenase enzymes in the mitochondria. The mitochondrial complexes create a gradient of hydrogen ions and hence an electric potential called proton-motive-force. This potential drives the phosphorylation of adenosine diphosphate (ADP) by ATP-synthase enzyme to new adenosine triphosphate (ATP). Therefore, the protein bound states of NADH are utilized for the generation of ATP, and this association suggests that the comparison of free and bound states of NADH can provide information on the metabolic state of the cell as a whole

[2, 8, 10]. It has been reported that the close adherence of O157:H7 serotype of *Escherichia coli* to the epithelium of the gut causes the brush border microvilli to undergo effacement, the process of attaching and effacing [63]. It triggers actin-rich pedestal formation underneath the bacteria. It is also found that *E. coli* can cause activation of apoptosis in numerous epithelial cell lines however mechanisms are still unknown. These reports point out the importance of characterization of the cell metabolism in case of a bacterial infection. NADH in free form at room temperature in aqueous solution has a mean fluorescence lifetime of ~ 0.4 ns; although protein bound NADH has an increased value of ~ 2.5 ns which is significantly high [1]. The reduction in lifetime is attributed to adenine present in the free folded forms which subject the nicotinamide molecules to self-quenching, consequently preventing it from spreading on the substrate. As mentioned earlier, the bound NADH forms are mostly attached with membrane protein complexes of the mitochondria and hence involved in ATP generation. Studying these mechanisms can help us better understand the bioenergetics of cell culture and accurate characterization of free and bound alterations in normal state and the range of these alterations too [64]. The application of FLIM helps us investigate cellular metabolism in case of bacterial infection. A 3T3 cell culture was imaged for a duration of 4 h from

incubation of wild-type *E. coli* which displayed characteristic changes in metabolism explained by the comparison of relative contributions from the free and bound species and their respective lifetimes [63].

Jan Rupp et al. employed 2P-FLIM to visualise and analyse host–pathogen interactions during the intracellular stage of *Chlamydia trachomatis* infection [65]. The direct link of chlamydial and cellular NAD(P)H signalling pathways was demonstrated for the first time at high resolution in the spatial and temporal domains. Unlike previous studies that depended on analyses of metabolic gene expression using micro-array and RT-PCR techniques, this study enabled study of chlamydial metabolism independent of metabolism of the host cell. Again, Xiangming Meng et al. [66] reported a viscosity probe using lysosome targeted two photon fluorescent probe enabling more precise investigations into the process of autophagy. Conventional methods for monitoring autophagy include electron microscopy and western blotting, which are time consuming and expensive. Thus, 2P-FLIM technique provides novel and more precise measurements compared to the conventional techniques in understanding the infections and intracellular metabolic changes during the same.

4 Two photon fluorescence lifetime microscopy in neuroscience

Two photon laser scanning microscopy has drastically altered our viewpoint on the kinetics of molecules in the cells and dynamics of physiological as well as pathological processes in the organs of the nervous and immune system [67, 68]. This in turn, makes it convenient for the scientists to focus more on the development of novel diagnostic and therapeutic tools. The near-infrared short-pulsed excitation is significant as it enables in-depth tracking of cell motility in organisms, non-invasively. 2P-FLIM has been used extensively to study functional aspects of the cell at a molecular level, like protein–protein interactions [2, 13]. The techniques that enable diffraction limited FLIM based on TCSPC systems can be considered as most meticulous. The transport of oxygen causes a possible hindrance on the ability of the brain to meet variable metabolic demands, although diffusion of oxygen in the cerebral cortex is yet to be directly investigated. Concurrent imaging of two photon fluorescence assisted by endogenous NADH as well as the microcirculation of the cortex illustrates transparent boundaries of diffusion of oxygen in the cortical tissue of mouse. The NADH auto-fluorescence shows a rapid increase over a narrow partial pressure of oxygen (pO_2) range with a $p50$ of 3.4 ± 0.6 mm Hg, which establishes a virtual binary reporter of hypoxia [69]. The NADH auto-fluorescence illustrate exceptionally delineated geometrical patterns in transient

cortical tissue boundaries. This defines the limit of tissue oxygen diffusion from the microcirculation in the cortex and exhibits an exceptional similarity to the ideal Krogh tissue cylinder. The micro-vessel fabrication to deliver oxygen demonstrates the penetrating arterioles as primary sources of oxygen as well as the capillary network. This provides confirmation to the fact that the cortical oxygen fields exist with sharp micro-regional gradients of oxygen. Therefore, two photon NADH auto-fluorescence imaging can be implemented to illustrate vascular supply regions and localization of functional micro-regional cortical hypoxia with a spatial resolution in the micro-meter range. NADH in tissue boundaries show a definitive cylindrical geometry and circumscribe coaxial central blood vessels. There is an infinitely small probability that those distinct vascular and tissue geometries have emerged by chance and their striking similarity to the ideal Krogh tissue cylinder exhibits an elemental relationship to tissue oxygen diffusion. The fluorescence lifetimes of NADH in the vascular supply provide a biophysical interpretation of the NADH patterns at the tissue boundaries. This enables the localization of the vascular region with cortical hypoxia.

Disruption of cerebral functions due to alterations in energy metabolism are significant in the onset of neurodegenerative diseases and other pathological conditions. In a study, the model for inducing Parkinsonism involved PC12 cells that were treated with neural growth factor (NGF) and the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is not cytotoxic, but was administered in its active form—MPP+ [70, 71]. Parkinson's disease is a very prominent neurodegenerative disease affecting dopaminergic neurons in the Substantia Nigra pars compacta (SNpc) of the brain—lowering dopamine levels in the brain and consequently impairing motor co-ordination [72]. Several studies have hinted towards dysfunction of complex I of the mitochondria leading to depletion of energy in neurons [73]. In the physiological state, the conversion of MPTP to MPP+ is carried out by the enzyme monoamine oxidase B (MAO B) after it crosses the blood brain barrier. MPP+ is then selectively taken up by dopaminergic neurons and further it accumulates in the mitochondria of SNpc neurons, damaging the complex I in the mitochondria [70].

Time-resolved 2P-FLIM measurements were used to quantify the ratios between reduced and oxidized states of NADH and FAD, and the ratios of free and protein bound states of the same [72, 74]. The excitation wavelengths for two photon and the emission filters were set at 760 nm and 447/60 nm for NADH and 860 nm and 525/60 nm for FAD, respectively. The double exponential function was used to quantify lifetime components of NADH and FAD:

$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}$$

where τ_1 , τ_2 and a_1 , a_2 are the short and long lifetimes and their relative amplitudes, respectively.

The change in NADH auto-fluorescence lifetimes were observed with dosage of MPP+ (Fig. 3). Cells treated with 1000 μM of MPP+ showed a decrease in average lifetime, τ_{avg} of 17%, tested for significance from ANOVA. Reduction of lifetimes in free and protein bound FAD were also observed, but the difference was smaller when compared to NADH. Ratio of contribution from free to protein-bound FAD decreased by 27% relative to control, demonstrating an increase in the protein-bound component of FAD. In conclusion, measurement of NADH and FAD lifetimes in free and bound states serve as indicator for Parkinson model in PC12 cells treated with MPP+. To investigate further into the complex alterations along the progression in Alzheimer's disease (AD), in vivo imaging is performed in preclinical trials in murine models [67]. The modifications in the endogenous fluorescence of NADH and its variations in AD has been measured by utilizing 2P-FLIM microscopy and the phasor analysis method. The AD related alterations to mitochondrial functions are also determined by measuring the levels of NADH in the cortex of the AD murine models.

To better understand the regulation of biochemical signals induced by neural activity, it is mandatory to measure protein–protein interactions and activity of enzymes in micro-compartments of neurons such as dendrites and their spines, axons etc. [75, 76]. The use of 2P-FLIM is increasingly familiar in the domain of measurement of protein–protein interactions using FRET at the level of spines in intact tissue. Lifetime measurement enables the direct measurement of the ratio of donor bound to acceptor even with small number of signal photons. Of the numerous Ras sensors that have been reported through previous studies, Raichu is quintessential,

consisting of a fusion of ECFP, anchored in plasma membrane, RBD, Ras and EYFP. Due to the dim ECFP fluorescence and the minute signal generation upon activation of Ras, Raichu was not suitable for 2P-FLIM measurements in small compartments. A rapid, increased high-sensitivity Ras sensor (FRas-F) optimized for 2P-FLIM measurements was developed for small neuronal compartments. After tagging RBD with mRFPs, B20% additional signal was obtained and therefore 40% photons were required to obtain a significant signal-to-noise ratio. The affinity between Ras and RBD was reduced and the sensor became reversible with rapid decay kinetics (Fig. 4a) [76].

The fluorescence lifetime of a specific molecule such as NADH is constantly scrutinized under specific characterized micro-environments, but nevertheless with an extensively increased metabolic activity of a particular component like the brain, it will definitely have several different implications in NADH lifetime. The application of fluorescence lifetime combined with correlated anisotropic measurement conclude that NADH can exhibit numerous lifetimes [101]. The diversified populations of NADH illustrating several lifetimes is referred to as different species of NADH which includes unbound and bound NADH (enzymes of different sizes) in various conformations, folded or extended. This exhibited results which can be concluded with the fact that measurements of NADH lifetimes can range from ~ 150 ps to ~ 600 ps. The difference in the lifetimes can be due to the changes in specific factors such as temperature, viscosity, and pH. However, the enzymatic binding may be primarily responsible for the differences. Further it can be concluded that enzymatic binding is in direct relation to NADH cycling in pathways that produce energy. To investigate the difference in lifetime of NADH in rat hippocampus and human

Fig. 3 Micrographs denoting average lifetime of NADH by pseudocolor mapping in PC12 cells that were **a** differentiated and untreated; and cells treated with **b** 50, **c** 100, **d** 250, **e** 500, and **f** 1000 μM of MPP+. Colour bar represents the range of average fluorescence lifetime. Figure reproduced (from Ref. [71]) with kind permission from Springer Nature. (Color figure online)

Fig. 4 **a** Change in lifetime of NADH after treatment of KCL. **b** Time course of the activation of Ras measured as the binding fraction of mEGFP-Ras to RBD-mRFP. **c** Western blot representing the total Ras in the lysate activity of endogenous Ras pulled down with GST-RBD beads. The time scale represents elapsed time after KCl application (min). **d** Time course of endogenous Ras activity. Figure is reproduced (from Ref. [76]) with kind permission from Nature

cortex, 2P-FLIM was used. Metabolism in Astrocytes play a crucial role in aiding the proper functioning of neurons. In case of any neurological diseases (e.g. Human temporal lobe epilepsy), exorbitant alterations are noticed due to the imbalance in the metabolism among these specific cell types [76, 77]. However, the extent to which these metabolic imbalances are duplicated in several animal models remains argumentative. Two groups of control and epileptic pilocarpine-treated rats were used to check the metabolic responses after stimulation to detect any variations in NADH binding and distribution that would be conclusive of metabolic imbalance. The differences in metabolism between control tissue and pilocarpine-treated tissue should evidently be significant when the neural activation is increased by bicuculline stimulation. The two-component decay fit method resulted in identification of the unique NADH lifetimes in rat and human brain. NADH concentrations are reduced under rest for the pilocarpine-treated tissue when compared to the control tissue. Therefore, it can be concluded that NADH concentration reduction in the pilocarpine-treated rat could be indicative of a decreased metabolic potential of the neurons and astrocytes, along with a quantitative overall loss of neurons, which is regarded as a hallmark of epilepsy. Also, as expected, the total NADH concentration rises after stimulation. In case of control rat, the total increase in the concentration of NADH is almost steady between the cell and dendritic layers. This is indicative of the fact that the NADH

distribution between free and bound species remains consistent in the dendritic layer, but in the cellular layer it favours the bound species. The total NADH concentration shows a steep rise in the dendritic layer when compared to control tissue. However, the cell layer from pilocarpine-treated rats indicates minute changes as compared to control rats or the dendritic layer of pilocarpine-treated rats. The variations in NADH distribution when stimulated indicate metabolic imbalance or dysfunction which is suggestive of the conglomeration of NADH. This may suggest a decreased oxidative metabolism in this tissue. The correlation between the degree of NADH binding and the enzymatic and metabolic load should be ideal. The comparatively shorter-lifetime bound NADH can be seen in neutrophils, which is conventional since the majority of dendritic synapses are mostly located there and have increased metabolic demand than the cytosolic counterpart (unbound NADH). A limitation for these studies is that it is still not clear about the physiological state of the tissue during the acquisition of the image. However, this limitation can be partially counteracted by the increased photon collection time which will give rise to temporal averaging of neural activity at the site of the image. Further studies will be attempted to correlate between measured NADH lifetimes within the analysed physiological conditions.

The molecular mechanisms of energy metabolism in the cerebrum are intricate. Identification and characterization

of these mechanisms play a pivotal role in improving our understanding of the functional and pathological alterations in the brain. NADH is an electron carrier which is ubiquitously produced, and is critical to the function of metabolic pathways including glycolysis and oxidative metabolism. Measuring its auto-fluorescence acts as an indicator of its metabolic activity and has been proven to be minimally invasive till date [78, 79]. (Figure 5) [27, 32]. More recently integration of two photon fluorescence microscopy with

time-resolved fluorescence lifetime imaging microscopy extends the utilization of NADH measurement auto-fluorescence to improve the characterization of the fluorophore in micro-environmental conditions.

In conclusion, neuroscience has always been a complex field of research due to the intricacies of the nervous system. Two photon fluorescence microscopy paves the path for advanced imaging techniques for the brain as well as in vivo experiments which deliver accurate results. The

Fig. 5 **a** Two-photon imaging portion of the in vivo imaging system, modified for FLIM measurements. SH: shutter; P: polarizer; M: reflecting mirror; EOM: electro-optic modulator; DM: Dichroic Mirror; XY: galvanometer-based scanners; PMT: photomultiplier tube; PCM: photon counting module. Inset: the sealed cranial window model represented in a rectangular image with measurements, approximately 3 mm in diameter at 570 nm. **b** In vivo image of a cortical astrocyte showing NADH auto-fluorescence, with **c** pixel-wide distribution of 2-component fit clearly showing 4 distinct peaks

performed with commercial SPCImage software. **d** Example in vivo cortical NADH fluorescence decay for 4-component lifetime fit, computed with custom software. Blue profile: Collected, binned photon counts, Green profile: computed IRF, Black-dashed lines: fitting boundaries, Red profile: Computed decay profile **e** Computed 2, 3, and 4 component lifetime residuals fit to the example in vivo lifetime data, with reduced χ^2 errors of 195.16, 2.64, and 2.54, respectively. Figure reproduced (from Ref. [32]) with kind permission from OSA. (Color figure online)

summarization of papers dealing with 2P-FLIM has shown precise results in experiments which can be achieved with high reproducibility. Due to its minimally-invasive nature, it has been widely accepted and shows a great promise in the novel path of scientific improvements.

5 Two photon fluorescence lifetime imaging in cancer research

Metabolic changes in the body during tumorigenesis are one of the hallmarks in cancer [80]. Therefore, in case of carcinogenesis, the microenvironment of the metabolic electron carriers as well as relative amounts of NADH and FAD can be monitored non-invasively with appropriate metabolic imaging techniques [81]. A significant increase in the nuclear to cytoplasmic ratio (NCR) in relation to depth in normal tissue epithelia has been reported. However, there appears to have minute changes, which can not be considered significant in the same analysis in precancerous tissues. The significant reduction in the redox ratio of substantially less differentiated basal epithelia in comparison to the mature cells present in the superficial layer of normal stratified squamous epithelium indicates a direct relation of cells with high NCR and metabolic activity. In certain cases, there lay a significant reduction in the contribution of protein-bound NADH lifetime relative to normal epithelial tissues, in both low and high-grade epithelial precancerous. However, a significant increase in the lifetime but decreased contribution in intensity of protein-bound FAD were observed exclusively in precancerous tissue samples of high-grade. A high level of intracellular variability was observed regarding the NADH, FAD fluorescence lifetimes and redox ratio in precancerous cells in comparison to normal cells. Biochemical estimation of the concentration of NADH is crucial in determining the cellular metabolic activities. The NADH/NAD⁺ reduction–oxidation pair points directly to the electron transfer system of the mitochondria. Measurement of the fluorescence lifetimes and the relative contributions of free and protein-bound NADH using 2P-FLIM helps in the derivation of functional maps of intracellular reduction–oxidation ratio [19]. A significant reduction was observed in the fluorescence lifetime of both states of NADH, free and protein-bound, by systematic analysis performed on the control cells. There also appeared to be significantly less contribution by protein bound state of NADH as the progression of the cells took place from an early growth phase to the logarithmic and eventually to the confluent phase. The treatment of the cells with potassium cyanide (KCN) combined with the serum starvation provided comparable changes [19]. Therefore, it was hypothesized that the imposed conditions caused an increase in the NADH/NAD⁺ ratio. In support of the above stated hypothesis, there are several justifications which point

directly to the coenzyme NADH as the determinant for the indicated fluorescence measurements. According to reports, breast epithelial cells (MCF10A) transfected with an empty vector (pZIP) consist of three major fluorophores: NADH, FAD and tryptophan. The KCN addition to the cells causes inhibition of the electron transport system, directly influencing the ratio NADH/NAD⁺ ratio. Consequently, this causes the increase in NADH fluorescence intensity of fluorescence by a factor of 1.3, consistent with the previous reports [19]. Finally, the fluorescence lifetimes measured on an average in the two-fluorescence emission in the MCF10A pZIP cells are also consistent with the literature reports of the free and bound NADH. It seems highly unlikely that tryptophan will have a contribution in the fluorescence emission at two-photon excitation wavelength (~740 nm), since it consists of a single-photon absorption peak of 280 nm. Increase in cell confluency, in turn causes increase in cell crowding, therefore depleting oxygen consumption. On the other hand, an increased NADH/NAD⁺ ratio is expected due to reduced oxygen consumption. Therefore, it can be concluded that the reduction in contribution of protein-bound NADH and fluorescence lifetime of both the free and protein-bound NADH and with increasing cell confluence is significantly associated with an increase in the ratio of NADH/NAD⁺.

Cancer metastasis consists of sophisticated cell to cell interaction and behaviour with the extracellular matrix and cells with increased metabolic activity. Multiphoton microscopy (MPM) enables deep tissue imaging with reduced toxicity when compared to other optical methodologies in order to observe the invasion and metastasis within the cell. MPM when combined with second harmonic generation (SHG), FLIM and spectral-lifetime imaging microscopy (SLIM) causes facilitation in imaging of collagen present in the stroma as well as the interactions of the tumour and the stroma [29, 82]. This observation also brings to light the intricate architecture as well as the remodelling of the microenvironment around the tumour. FLIM enables the identification and characterization of fluorophores, both exogenous and endogenous in nature, for example FAD and NADH metabolites to determine the metabolic state and exhibit optical biomarkers [83]. SLIM allows in extensive identification of endogenous and exogenous fluorophores while simultaneously collecting their spectra and fluorescent lifetimes, thus coming up with an optical molecular “fingerprint”. Therefore, further investigation into these approaches develops the insights by which tumour progression, invasion and metastasis can be visualized as well as quantified. Multiphoton microscopy, more specifically multiphoton FLIM, is in fact appropriate and suited for detection of alterations in metabolic enzymes [63, 69]. It can also be used to differentiate between normal and cancerous epithelial tissues [14]. In an another study, it was found that increased lifetimes of free and protein-bound FAD associated with invasive metastatic

mammary carcinoma cells. The results may be attributed to increased rate of glycolysis causing an increase in the NADH concentration [84]. It is shown previously that high rate of glycolysis can also cause an increase in the synthesis of lactic acid. This causes protons to be released in high amounts into the extracellular space, thus reducing the pH, promoting matrix degradation and local invasion [14]. The changes in cellular metabolism are relevant in their association with tumorigenesis and progression, and quantifying them thus represents a potential for better diagnostic methods.

In the United States of America, prostate cancers (PCa) is one of the major types of cancer in males [85, 86]. A significant limitation in the therapeutic routine is the lack of experimental methods to predict the response of therapy strategies. One strategy of cancer cells in inhibiting apoptosis is by regulating ROS production and intracellular signaling, which is effected by dysfunctions in the mitochondria such as unregulated oxidative phosphorylation. These are promising targets for therapeutic treatments. A 3-channel FLIM assay and quantitative analysis techniques were used in a recent study to assess the action of anti-cancer drug doxorubicin in PCa cells. Auto-fluorescent intracellular metabolites of NAD(P)H, FAD and aromatic amino acid Trp were tracked by their lifetimes. The metabolic state of the mitochondria was characterized in pre and post treatment conditions, using enzyme-bound forms of the fluorophores as markers (Fig. 6) [86]. The mean lifetime and contribution by enzyme-bound lifetime of NAD(P)H was increased while the contribution by enzyme-bound fraction in the case of FAD is reduced. FLIM-based redox ratio of NAD(P)H/FAD and intracellular ROS increased, which was

followed by induced apoptotic activity (Fig. 6). This was the first study where a FRET assay in PCa cells showed Trp-quenching, attributed to Trp-NAD(P)H interactions, displaying the parameter of correlating energy transfer efficiencies and redox ratio to be sensitive enough in predicting drug response [86]. In conclusion, this application of FLIM can help make significant advancements in early detection of therapeutic action in prostate cancer.

6 Two photon fluorescence lifetime microscopy in wound healing

With average area coverage of 20 sq.ft, the skin is the largest organ of our body, externally as well as internally. It also acts as the first line of defence against toxicity and pathogens. Normally, the skin can heal any external damage caused but however, wounds can become fatal if they aren't taken proper care of. In the field of plastic reconstructive surgery, wounds have proven to be one of the major causes of trauma. Therefore, appropriate diagnosis is important for accurate treatment [87, 88]. Conventionally, wound diagnosis relies on the expertise of particular physicians based on the non-quantitative clinical signs, or histochemical assays performed using invasive methods of biopsies [89–91]. With the recent advances in science, following the effective methods of light matter interaction it is possible to attain appropriate diagnosis of a tissue, quantitatively and real time in a non-invasive procedure simply based on the imaging [92]. Through the various methods of absorption, scattering or fluorescence, depending on the nature of interaction, it enables scientists to

Fig. 6 PCa cells, upon doxorubicin treatment show decrease in the enzyme-bound fraction of FAD- a_1 %. Upper panel **a–f** LNCaP and lower panel **g–l** E006AA (AA) cells. PCa cells: LNCaPs ($n=26$) with 4400–4890 ROIs, and E006AA cells ($n=28$) with 5585–6372 ROIs, each ROI of 2×2 pixels, were studied for 5 time points (0, 15, 30, 45 and 60 min). The representative **a–e** and **g–k** color coded images and

f and **l** the histograms showed upon treatment with $1 \mu\text{M}$ doxorubicin there is a decrease in the enzyme-bound fraction of FAD- a_1 % with time (0–60 min) when compared with untreated 0 min time point control (Ctrl). Figure reproduced (from Ref. [86]) with kind permission from Springer Nature. (Color figure online)

observe the structural or molecular components of tissue sections. Multiphoton laser scanning technique improves resolution as well as penetration depth and has a greater advantage over macro-optical modalities. The optical sectioning ability is devoid of a pinhole, because it lessens the alignment difficulty as well as photo bleaching. On the top of that, the near infra-red (NIR) excitation wavelengths have proven to have extended the imaging of the deep tissue imaging limit up to 2 mm [93, 94]. The most usual multiphoton techniques in tissue imaging are two-photon fluorescence microscope (2PF) and SHG imaging. In 2PF, the simultaneous absorption of two photon by the fluorophores caused excitation to a higher energy real state before it starts emitting the fluorescence. While in SHG, there are two photons of identically similar energy combines forming a new photon, thus doubling the energy of the incident photon [95, 96].

To characterize epithelia morphology *in vivo* as well as *ex vivo* in animal and human tissues involving fresh biopsies, cellular NADH auto-fluorescence in two-photon modality has been used as marker [87, 97, 98]. Several epidermal layers of skin were resolved *in vivo* at subcellular spatial resolution with the use of 2PF imaging based on the morphology of the cellular features [82, 99, 100]. In addition to that, the technique involving TCSPC system in combination with 2PF has enabled us to provide functional imaging by measuring fluorophore lifetime. FLIM is one of the most effective methods to calculate the metabolism of the cell real time, *in vivo*. This can be achieved by calculating the fluorescence lifetime decay of NADH or FAD. The cells which are present in the basal layer provide the most prominent metabolic change in its activities, while on the other hand cells on the epidermal surface layers are observed to have lower metabolic activities in comparison. FLIM has proven to be capable of characterizing the epithelial tissues which are crucial in wound healing as well as other pathological conditions [81].

NADH is a metabolic coenzyme and is associated with the metabolic activities of the cell, primarily involved the electron transport chain (ETC) of oxidative phosphorylation. The fluorescence emission spectra of the NADH in free and bound forms fall in a short range of values and come within a very narrow band. Although this observation is true to an extent but when NADH is bound to a protein, it is observed that the fluorescence lifetime increases from ~0.4 to ~2.5 ns. Therefore, to predict the relative concentration of the individual states, the contribution of the functional free and bound states in an associated double exponential lifetime combined has to be evaluated. It has also been observed that higher concentration of bound NADH can be found in higher cellular metabolic activity as and when compared to lower cellular metabolic activity. Additionally, the ratio of NADH bound form to FAD bound form can be a marker for

the determination of the relative metabolic activity and is termed as cellular redox ratio [14, 81].

It was reported that with the help of 2P-FLIM and SHG microscopy, the evaluation of wound healing is performed by inducing an artificial incision wound using punch biopsy [87]. Following the day of formation of the wound, a 20-day time course has been observed for evaluation. The relative cellular metabolic activities are further evaluated with the progression of time by the exhibition of amount of NADH bound to free ratio. On the other hand, the fluctuations in the concentration of collagen are correlated with the SH intensity. The final evaluation results indicate that the wounded sites in the inflammatory and granulation phases have increased metabolic activities and it gradually decreased with the healing of the wound. It is noted that in the initial stages of healing, the intensity of SHG is reduced (or collagen concentration) which indicates that the collagen has been degrading during the migration of the cell. Also, with the renewal in the formation of collagens, SHG signal shows a gradual increase. In most cases, the wound healing takes place from the edges of the wounded area towards the center, therefore it is observed that the metabolic activities are elevated at the edge in the initial stages which marks the higher value of bound and free form of NADH ratio in the measurement of fluorescence lifetime [101]. In addition to that, in the proliferative phase, the center of the wound shows increased metabolic activity in comparison to the edge. This happens because at this stage, the edge is going through the remodelling phase, thus decreasing the cellular activity and also the deposition of collagen in the wound gap for filling. This also causes a marked increase in the intensity of SHG. The proliferative phase is followed by filling the whole wound with granulation tissues which mainly consists of collagen and the metabolic activity starts decreasing gradually [102]. This leads to the formation of a scar, which is primarily composed of connective tissues indicating the healing of the wound. It is also marked by increased SHG signal intensity than when compared to a normal tissue. The blood influx in the scar tissue is highly reduced due to the lack of cells which eventually leads to apoptosis of the blood vessels. The scar is eventually left with tissues having low metabolic activity and a high deposition of collagen.

Damage in the corneal epithelial cells can have extensive consequences leading to infection as well as the consecutive destruction of the stroma in the cornea, eventually leading to loss of vision [103]. To stop the process from extending its possibility to such grave consequences, early and accurate diagnosis is a necessity. In the advent of several optical methodologies, optical coherence tomography and confocal laser scanning microscopy have been established to have faster and proven results, based on information collected through non-invasive techniques and high-powered reflectance microscopy. However, compared to these techniques,

two-photon fluorescence microscopy (2PF) provides better technology due to the increased penetration depths of more than 1 mm, high resolution below 1 micrometres as well as restricted photo-bleaching and photo damage due to the excitation of the two-photon only in the focal area. 2PF microscopy aims to analyse the changes in metabolism and fluorescence lifetimes of NAD(P)H, in both free and bound forms, during wound healing of corneal epithelium because in this particular context it is regarded as the most important metabolic cofactor. Alteration of temperature and the specific inhibition of oxidative phosphorylation in the cell combined with the fluorescence intensities of the NAD(P)H were analysed to detect the process of wound healing. The decrease in the temperature and rotenone induced inhibition of oxidative phosphorylation causes alterations in the cell auto-fluorescence; rotenone causes prevention of NAD(P)H oxidation which increases the levels of fluorescence intensity. This also causes the upregulation of glycolysis which is due to the decrease in the number of NAD(P)H binding to the proteins, in turn decreasing the overall fluorescence lifetime. This also causes an increased ratio of free and bound NADH. Reduction in temperature in short terms shows lower levels of enzymatic activity but with higher levels of protein binding and thereby increasing lifetimes and decreased ratios of free to bound NADH. Moderate temperature maintains the optimum levels of enzymatic activity and thus enhancing metabolic activities of the cell; therefore increased glycolysis. The fluorescence intensities show no drastic alteration which concludes that the potential of FLIM use in this context is feasible and that it is a highly sensitive method. As far as 2P-FLIM is concerned, it is a non-invasive method and by detecting the fluorescence lifetime changes in the ocular surface during corneal epithelial wound healing. Additionally, a suitable setup for in vivo imaging of the eye combined with extensive analysis of the metabolic changes in the different compartments of the cell with the functioning metabolites will extend the possibilities of gaining more insight in a single cell level. It can also be utilized in development of long-term therapeutic strategies and in determining the effects of novel substances on metabolic activities of the cell in pre-clinical and clinical trials.

Wound healing in cutaneous tissues, especially in cases of chronic wounds is characterized by prolonged and excessive inflammation followed by formation of callus and infection. The wound shows improper vascularity due to the endothelial inflammation. The current therapies target an extensive clinical success but there is no exact therapy or development of biomarkers to determine and evaluate the status of these wounds. Two photon excited fluorescence (2PF) has shown numerous advantages in depth evaluation and collection of signal. The measurements of intrinsic fluorescence of NADH and FAD, an optical redox ratio, eventually find out the relative rates of glucose metabolism and oxidative

phosphorylation increase the quality of prognosis. Label free multiphoton microscopy assesses the initial quantitation of the metabolic biomarkers in diabetic and non-diabetic patients [104]. In a study, the optical redox ratio of NADH and FAD fluorescence shows significant differences at day 0 (normal tissue) with no delineation between the epidermis and dermis and at day 3 (wounded tissue) with an evidence of hyperplastic epidermis at the edge of the wound. By day 10, there is a shift from anabolic proliferative phase to the migratory phase in non-diabetic epithelial tissue acted on by ATP dependent actin reorganization as shown in Fig. 7. In diabetic epithelial tissue, the wounded edges are significantly lower with increased proliferation of keratinocytes and absence of non-healing edges characterized specifically by chronic wounds. In fact, hypoxia induced and increased glucose catabolism in mice has been observed in tissues with low redox ratios. Granulation tissues show increased redox ratios, correlated to accumulation of neutrophils and density of blood vessels, production of reactive oxygen species. This evidently depicts the metabolic differences in the epithelium and granulation tissues, thereby demonstrating the fact that depth resolved and high-resolution optical imaging techniques are crucial in accurate diagnosis of wound metabolism [101]. This would further help in investigating metabolic biomarkers in characterization of delayed wound healing. Alterations in the measurement of optical redox ratio and NADH fluorescence lifetime trigger sensitivity in keratinocyte functions at the edge of the wound and varied metabolic activity in diabetic wounds.

In conclusion, wound healing follows a sequence of specific events and is regarded as an important physiological process. Identification and characterization of certain cells contributing to the process with their specific migratory patterns entails the process of wound healing progression. Further investigation, both qualitative and quantitative, provides crucial insights about the process in general. The versatility of optical methodologies on a scale of multiple components along with their non-invasive nature provide extensive future prospects, specially in case of clinical implications as well as cutaneous healing and skin tissue regeneration [105–107]. With the advancement of scientific research techniques, optical modalities have come a long way in determination and assessment of specific wound healing complications. While some of the modalities are simpler and easier and have cheaper investment on first line diagnosis, there exist more complex techniques which enable more sophisticated and focused information, in terms of structure as well as resolution. Judicial combination of several contrasts from numerous skin components provides a wide variety of skin related complications. Inclusion of several techniques such as dermascope for observing the morphology or identification of molecules using Raman spectroscopy, each one of the methods provides information which is unique to

Fig. 7 Optical ratio measurement at wound edge in (a–c) nondiabetic and (e–g) diabetic mice on (a, e) day 0, (b, f) day 3, and (c, g) day 10 after wounding. (d) Spatial variability in the redox ratio enables discrimination of wound regions similar to H&E staining. (h) Average redox ratios at different time points from the epidermis and granula-

tion tissue (GT) demonstrate significant changes in epithelial metabolism. (b–c, f–g) Decreased redox ratio at day 3 and thickening by day 10 is evident in both groups. Figure reproduced (from Ref. [104]) with kind permission from ELSEVIER

a particular process but at the same time can be regarded as complimentary information. Multimodal multiphoton microscopy provides the most clinically accurate and sophisticated insight to wound healing study. Integration of numerous contrast mechanisms enables quicker, better quantitative as well as qualitative assessment of the process in progress. Specifically, in the case of SHG and 2P-FLIM, the overall assessment in the study is more favourable due to the increased penetration depth and resolution as well as 3D imaging which provides real time molecular activity in terms of structure and function.

7 Conclusion

2P-FLIM is a novel technique with potent applications in the biomedical field. There are, however, some challenges with the technique. Highly reliable FLIM statistical analysis can be performed when there is enough emitted photon count from the sample, which is unlikely in auto-fluorescent samples. Additionally, in biological samples, the probability of heterogeneous auto-fluorescent molecules within the same pixel is high, which makes the task of interpretation of data more tedious. Improvisations in the microscopy technique, combination of high sensitivity detectors such as hybrid GaAsP cathodes and enhanced analysing algorithms using

phasor plot which enables detection of different fluorophores in every pixel of the image frame with a lower threshold of photon count, can help address these issues. Constantly improving techniques and algorithms enable FLIM to be used a significant tool in clinical analysis for skin wound healing studies and cancer detection [105]. TCSPC enables the investigation of dynamic changes in metallic activity by acquiring data in time periods by repeated triggering of the sample [108, 109].

Significant changes in the metabolism and morphology of cancerous cell are key features of carcinogenesis. There is a shift in the metabolism of cancer cells from oxidative phosphorylation to aerobic glycolysis, resulting in an increase in the NADH concentration which is considered as a principle electron donor in a cells metabolism. Considerable changes can be observed in the extracellular matrix during cancer initiation and progression. Tumour cell behaviour is regulated by the extracellular matrix in the tumour microenvironment [68, 110]. Collagen which forms the scaffold of tumour micro-environment also influences tumour behaviour. 2P-FLIM are very useful for determining the metabolic characteristics of cancerous and normal cells as well as in case of tissue to identify the structure and function of dynamic and static living system.

2P-FLIM has also been employed in oncological research. Rapid and label-free detection of precancerous

and cancerous cells could be performed by 2P-FLIM, since they have a significantly higher fluorescence lifetime when compared to normal cells. This method was proved to be better than conventional staining and subsequent histological examination [111]. This technique was also applied to derive functional metabolic maps of MCF10A human breast cells by calculating the intracellular redox ratios in vivo, measuring lifetimes and ratio of free and protein-bound NADH in normal and stress conditions [19]. However, recent data has shown that fluorescence lifetime can derive even more data on the excited state dynamics and rotational mobility when combined with the measurement of anisotropy decays [2, 21, 112, 113]. The fluorescence anisotropy value is more in case of bound-NADH as compare to free-NADH and hence sensitive to discriminate them. The perspective of multimodal FLIM with CARS [114] and high-resolution microscopy systems including Stimulated Emission Depletion Microscopy (STED) [114], and near-field microscopy [115] provide great potential.

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Compliance with ethical standards

Conflict of interest All authors declare to have no conflict of interests.

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