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Phasor FLIM metabolic mapping of stem cells and cancer cells in live tissues

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1. ABSTRACT

We use the phasor approach to fluorescence lifetime imaging and intrinsic biochemical fluorescence biomarkers in conjunction with image segmentation and the concept of cell phasor for deriving metabolic maps of cells and living tissues in vivo. In issues we identify and separate intrinsic fluorophores such as collagen, retinol, retinoic acid, porphyrin, flavins, free and bound nicotinamide adenine dinucleotide (NADH). Metabolic signatures of tissues are obtained by calculating the phasor fingerprint of single cells and by mapping the relative concentration of metabolites. This method detects small changes in metabolic signatures and redox states of cells. Phasor fingerprints of stem cells cluster according to their differentiation state in a living tissue such as the C. elegans germ line and the crypt base of small intestine and colon. Phasor FLIM provides a label-free and fit-free sensitive method to identify metabolic states of cells and to classify stem cells, normal differentiated cells and cancer cells both in vitro and in a live tissue. Our method could identify symmetric and asymmetric divisions, predict cell fate and identify pre-cancer stages *in vivo*. This method is a promising non-invasive optical tool for monitoring metabolic pathways during differentiation and carcinogenesis, for cell sorting and high throughput screening.

2. Introduction

Stem cells and cancer cells have a unique metabolic signature associated with their phenotype. Metabolic patterns and changes are known to play an important role in stem cell differentiation and cancerogenesis (Wang 2009; Yanes 2010). The role of metabolic oxidation, oxidative stress and redox state has emerged as an important modulator of stem cell fate decisions such as self-renewal/pluripotency, differentiation/lineage-specification, programmed cell death and quiescence. (Smith 2000; Guo 2004; Ito 2004; Ogasawara 2009; Pervaiz 2009; Hamanaka 2010; Yanes 2010). Oxygen levels regulate mitochondrial metabolism, intracellular generation of ROS and ultimately the differentiation of stem cells (Balaban 2005; Simon 2008). ROS are produced by the mitochondria during oxidative metabolism and ROS levels influence metabolite concentration, transcription factor activity and other upstream signaling events involved in cell division, differentiation, survival and oncogenic transformation (Kamata 1999; Zhang 2002; Fjeld 2003; Hamanaka 2010).

Recently non-invasive optical techniques have been developed to obtain information on cell metabolism and discriminate between different states of cells and tissues (13-20). These methods exploit intrinsic auto-fluorescence of cells and tissues and multi-photon microscopy. Multi-photon microscopy is suitable for high resolution and long term imaging of living tissues. It allows investigation of local environment in femtoliter volumes deep in tissues with minimal photo damage and phototoxicity (Denk 1990; Squirrell 1999; Zipfel 2003; Zipfel 2003). FLIM measures the time decay characteristics of the cell and tissue microenvironment and allows molecular localization and identification of intrinsic fluorophores and endogenous proteins such as collagen, elastin, porphyrin, retinoids, flavins, nicotinamide adenine dinucleotide, hemoglobin and serotonin. Some intrinsic fluorophores have a physiological relevance and can be used as biomarkers; for example reduced nicotinamide adenine dinucleotide (NADH) is an essential cofactor for oxidation–reduction (redox) reactions and oxidative phosphorylation and glycolysis. Biochemical estimation of NADH concentration has been extensively used to monitor cellular energy metabolism *in vivo* and FLIM has become a valuable technique to image life cells and to analyze metabolic states of

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cells. (Chance 1962; Bird 2005; Chorvat 2009). FLIM has been recently employed by (Guo 2008; Uchugonova 2008; König 2010) to image and to discriminate undifferentiated adult human stem cells from differentiated cells in vitro. We recently demonstrated that the phasor analysis of FLIM, is a very sensitive fit-free method to identify and distinguish different metabolic and differentiation states of germ cells in a living tissue (Stringari 2011).

Here we use the phasor approach to fluorescence lifetime microscopy (29, 30) which allows a straightforward interpretation of intrinsic fluorescence signal from living tissues and cells directly in terms of physiological relevant fluorophores. We provide images of molecular species by using their phasor fingerprints, without resolving and assigning exponential components to the fluorescence species. We measure and identify cellular and subcellular metabolism in stem cells and colon cancer cells. By image segmentation we calculate the phasor distribution and average phasor value of the entire cells, the mitochondria, cytoplasm and nuclei. We directly map the relative concentration of free and bound NADH in the tissue and cells. Our results show that it is possible to monitor the metabolic activity of cells in living tissues in a non-invasive way.

3. Results

Identifying cellular and tissue components using the phasor plot

Each chemical species has a specific location in the phasor plot that is determined by the intrinsic characteristics of its fluorescence decay. This characteristic phasor fingerprint is used here to identify individual components in a complex system such as a tissue. Fig.1a shows the phasor location of the most important intrinsic fluorophores. Their positions in the phasor plot are well defined and clearly separated one from the other. The majority of fluorophores have decay with multiple exponential components because of their conformational heterogeneity. The phasor position of GFP is near but not exactly on the universal circle (Fig. 1a) since the fluorescence decay of GFP is not single exponential (Hess 2003). Collagen has a very short lifetime with a broad distribution of decay components due to the intermolecular cross links (Bornstein 1966). Its phasor is located inside the universal circle and closed to the temporal zero. The phasor position of retinol in DMSO has a specific location which is different from the one of the retinoic acid in DMSO, in agreement with the measured multi-exponential decays (Bel'Kov 1990). Both FAD and free and bound NADH phasor position are located inside the phasor plot. Their lifetime is a combination of several exponential (Lakowicz 1992; König 2003; Schneckenburger 2004; Chia 2008). NADH has a different phasor position when binds with different enzymes such as lactate Malate dehydrogenase (MDH) and lactate deyhidrogenase (LDH) (Lakowicz 1992). The phasor position of protoporphyryn IX (in dimethylformamide and methanol) is located on the universal circle since it is characterized by a single lifetime component (Brancaleon 2004).

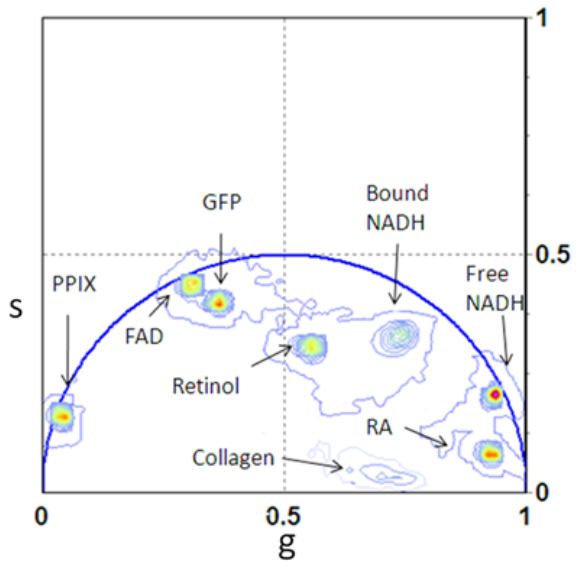

Fig 1. Shows phasors of pure chemical species identify tissue components. **(a)** Phasor location of pure chemical species. GFP in Tris buffer, Retinol in DMSO (pH 8.5), Retinoic acid in DMSO (pH 8.5), FAD in water (pH 7.4), free NADH in Mops buffer (pH 7), bound NADH in Mops buffer (pH 7) with Malate dehydrogenase (pH 7) and lactate dehydrogenase, Protoporphyrin IX in dimethylformamide:methanol (pH 7). **(b)** Intensity image of a semininiferous tubule from a mice expressing green fluorescent protein (GFP) from an Oct4 transgene. A chain of spermatogonial stem cells lie on the surface of the seminiferous tubule. **(c)** Phasor plot of the FLIM image acquired in b. The green and the blue cluster are located in the phasor position of pure GFP and pure collagen clusters.

Sensing cellular and sub cellular metabolism with Phasor FLIM

The average phasor is very sensitive to small differences of free/bound NADH ratio and senses small subcellular differences in metabolites. The average phasor of different cell compartments reveals a different concentration of free/bound NADH (Figure 2) in the cell nuclei and mitochondria. Cell average phasor, nuclei average phasor and mitochondria average phasor are located in separated areas of the phasor plot (Fig 2.e). Fig 2.e shows that cell nuclei have a higher ratio of free/bound NADH with respect to the mitochondria and the cytoplasm.

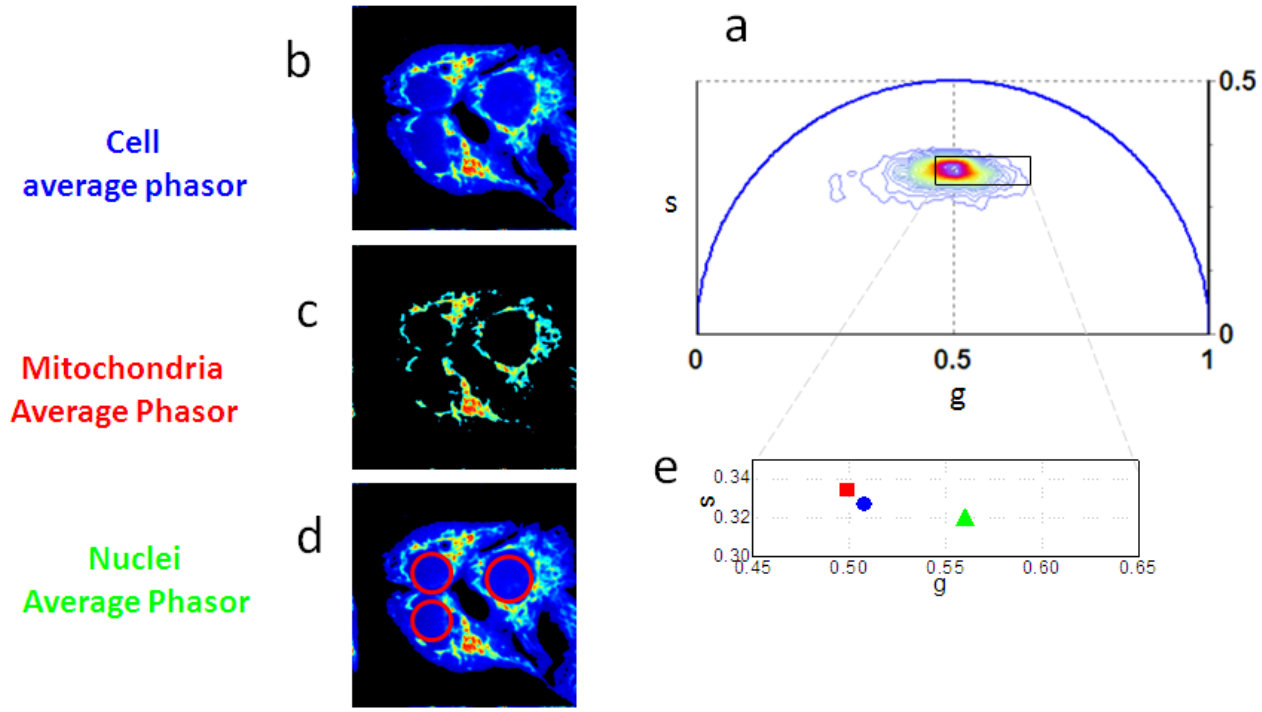

Fig. 2 Phasor FLIM sense subcellular metabolism.

(a) FLIM phasor histogram of the FLIM image excited at 740 nm from three colon cancer cells. (b) Fluorescence intensity image of the autofluorescence excited at 740nm from three colon cancer cells (c) the mitochondria in the cells are selected by intensity threshold (d) the nuclei of the cells are selected by image segmentation using a cursor of arbitrary shape. (e) zoomed area of the Phasor plot in Fig a. Average phasor values of cellular compartments are represented by a red square (average phasor of mitochondria), blue circle (average phasor of the entire cell) and the green triangle (average phasor of the nuclei).

Mapping the relative concentration of free and bound NADH ratio (Fig 3) allows visualizing the NADH/NAD+ ratio in the nucleus. Fig.3 shows that within the nucleus the NADH/NAD+ ratio is not homogeneous and there are regions with higher NADH/NAD+ ratios, that are localized is specific areas of the chromatin. Histone post-transcriptional modifications and epigenetic mechanisms are known to sense the variation of metabolites levels NADH/NAD+. (Zhang 2002; Fjeld 2003; Sahar 2009). Phasor FLIM would allow to map transcription territories via mapping the NADH/NAD+ ratio.

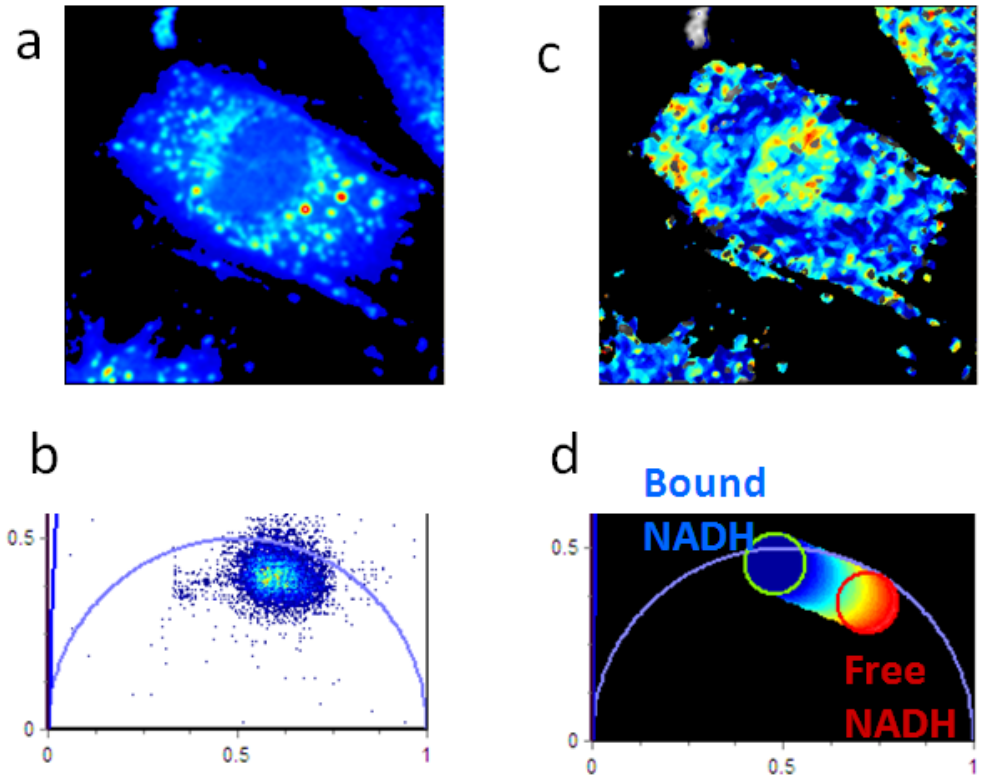

Figure 3. Free/bound NADH Heterogeneity in the nucleus of an undifferentiated myoblast cell: (a) Intensity image of the autofluorescence excited at 740nm from an undifferentiated myoblast cell. (b) FLIM phasor histogram of the FLIM image of the undifferentiated myoblast cell excited at 740 nm . (c) Phasor FLIM color map image representing the relative concentration of free to bound NADH according to the scale of the linear cursor in Fig.d. Within the nucleus there are island of higher values of bound/free NADH, i.e. of NAD+/NADH ratios. (d) Phasor plot selection using linear cluster combination that represents the relative contributions of free NADH (red-orange) and bound NADH (blue).

MATERIALS AND METHODS

Solution preparation: Retinol solution (Retinol all trans, Sigma no. R7632) was prepared in DMSO at a concentration of 1mg/ml at pH 8.5. Retinoic acid (Sigma no. R2625) solution was prepared in DMSO at a concentration of 0.01M (3mg/ml) at pH 8.5. 250 µM NADH (Sigma n.N8129) solution was prepared in 100mM Mops buffer at pH 7. A solution of 250 µM NADH is mixed 1:1 with 1000 unit/ml lactate dehydrogenase (LDH, Sigma no. L3916). A solution of 250 µM NADH is mixed 5:1 with ~700 units/mg protein of malate dehydrogenase (MDH, Sigma no. M1567). FAD (Sigma n.F6625) is diluted at 2 mg/ml in water at pH 7.4. GFP is diluted in 10mM Tris buffer at a concentration of 20 nM. Protoporphyrin IX (Sigma P8293) is diluted at 1.5 mg/ml in dimethylformamide:methanol (1:1) at pH 7. The phasor location of GFP and collagen were measured at 900 nm. The phasors of retinol, retinoic acid, NADH and FAD were measured at 760 nm and of porphyryn IX was measured at 790 nm wavelength. Collagen matrix is prepared with Collagen Type I (BD Biosciences 354236) at a concentration of 3.75mg/ml.

Colon cancer cells

Formation of stable cell lines that inducibly express dnLEF-1:DLD1 TR7 cells were transfected with a vector for Tet inducible dnLEF-1N (2g) by Effectene transfection reagent (QIAGEN). Stably transfected cells were selected in complete RPMI media containing 500ug/ml Zeocin (InvivoGen) and 10µg/ml Blasticidin (InvivoGen) and those

cells that were resistant to Zeocin and Blasticidin were isolated as single colonies. These clonal cells were expanded into individual cell lines and screened for the highest levels of induced dnLEF-1N protein expression by Western blot analysis.

Imaging. Fluorescence lifetime images are acquired with two different microscopes. The first set up is a two-photon microscope coupled with a Becker and Hickl 830 card (Becker and Hickl, Berlin). Ti:Sapphire laser system (Spectra-Physics Mai Tai) with 80 MHz repetition rate is used to excite the sample. The laser is coupled to an Zeiss Axiovert S100TV microscope. The scanning system is constituted by a scanning mirror (Cambridge Technology Mirror scanner 6350). A Zeiss 40 x 1.2 NA water immersion objective is used. For image acquisition the following settings are used: image size of 256 x 256 pixels, scan speed of 32 μs/pixel. A dichroic filter (700DCSPXR, Chroma Technologies) is used to separate the fluorescence signal from the laser light and the fluorescence is detected by a hybrid detector (HPM-100 of Hamamatsu). An additional barrier filter is used to block the near IR light. The second set up for FLIM is a Zeiss 710 microscope coupled to a Ti:Sapphire laser system (Spectra-Physics Mai Tai) and a ISS A320 FastFLIM (Colyer 2008). A 40 x 1.2 NA water immersion objective (Zeiss Korr C-Apochromat) is used. For image acquisition the following settings are used: image size of 256 x 256 pixels or 1024v1024 pixels and scan speed of 25 μs/pixel. A dichroic filter (690 nm) is used to separate the fluorescence signal from the laser light and the fluorescence. For the acquisition of FLIM images, fluorescence is detected by a photomultiplier (H7422P-40 of Hamamatsu) and a 610 nm short pass filter is placed in front of the detector. FLIM data are acquired and processed by the SimFCS software developed at the Laboratory of Fluorescence Dynamics. The excitation wavelengths used were 900 nm, 880nm and 740 nm. All samples are excited at 900 nm if not differently specified. An average power of about 5 mW was used to excite the live tissue. FLIM calibration of the system is performed by measuring the known lifetime of the fluorescein with a single exponential of 4.04 ns. FLIM data are collected until 100 counts in the brightest pixel of the image are acquired. Typically the acquisition time was of the order of few seconds.

Data Analysis and phasor transformation. Every pixel of the FLIM image is transformed in one pixel in the phasor plot. The components g (x-coordinate) and s (y-coordinate) of the phasor plot are respectively the real and imaginary part of the Fourier transform of the fluorescence impulse response. The coordinates g and s in the phasor plot are calculated from the fluorescence intensity decay of each pixel of the image by using the transformations defined in equations 1 and 2.

When fluorescence lifetime data are acquired in the time domain, the components g (x-coordinate) and s (y-coordinate) of the phasor plot are given by the following expressions:

$$g_{i,j}(\omega) = \frac{\int_0^\infty I_{i,j}(t)\cos(\omega t)dt}{\int_0^\infty I_{i,j}(t)dt} \quad (1)$$

$$s_{i,j}(\omega) = \frac{\int_0^\infty I_{i,j}(t)\sin(\omega t)dt}{\int_0^\infty I_{i,j}(t)dt} \quad (2)$$

where the indices i and j identify a pixel of the image and ω frequency ($\omega=2\pi f$)., where f is the laser repetition rate , i.e. 80 MHz in our experiment. All phasor plots are calculated at 80 MHz, i.e. the first harmonic of the laser repetition rate and for some cases for higher harmonics.
The phasor transformations of FLIM data acquired in the frequency domain at an angular modulation frequency ω are:

$$g_{i,j}(\omega) = m_{i,j}\cos\varphi_{i,j} \quad (3)$$

$$s_{i,j}(\omega) = m_{i,j}\sin\varphi_{i,j} \quad (4)$$

where $m_{i,j}$and $\varphi_{i,j}$are the modulation and the phase of the emission with respect to the excitation. Estimations of the lifetime in terms of the phase and modulation can be performed in each pixel by the following formulas [1,2]:

$$\tau_\varphi = \frac{1}{\omega}tan(\varphi) \quad (5)$$

$$\tau_m = \frac{1}{\omega}\sqrt{\left(\frac{1}{m^2}-1\right)} \quad (6)$$

In the case of a single exponential decay the two lifetimes obtained by the phase and by the modulation with equation (5) and (6) are equal, while for a multi exponential lifetime system the apparent lifetimes are different.

In the phasor plot if the decay is a single exponential $I(t) = Ae^{-t/\tau}$ the coordinates are given by:

$$g(\omega) = \frac{1}{1+(\omega\tau)^2} \quad (7)$$

$$s(\omega) = \frac{\omega\tau}{1+(\omega\tau)^2} \quad (8)$$

where τ is the lifetime of the decay and ω is the laser frequency. There is a direct relationship between a phasor location and lifetime. Every possible lifetime can be mapped into this universal representation of the decay (phasor plot). All possible single exponential lifetimes lie on the "universal circle" defined as the semicircle going from point (0, 0) to point (1, 0) with radius 1/2. Point (1, 0) corresponds to τ = 0, while point (0, 0) to $\tau = \infty$. In the phasor coordinates the single lifetime components add directly because the phasor follows the vector algebra. A mixture of two distinct single lifetime components, each of which lie separately on the single lifetime semicircle, does not lie on the semicircle. All the combination of two single exponential components must be along the line joining the two lifetime points. In a system with many single lifetime components the phasor coordinate g and s are described as:

$$g(\omega) = \sum_k \frac{h_k}{1+(\omega\tau_k)^2} \quad (9)$$

$$s(\omega) = \sum_k \frac{h_k\omega\tau_k}{1+(\omega\tau_k)^2} \quad (10)$$

where h_k is the intensity weighted fractional contribution of the single-exponential component with lifetime τ_k. The phasor location of the mixture of single-lifetimes is the intensity-weighted average of the contributions of each single-lifetime that lie separately on the semicircle.

In general in a system with multiple fluorescent components like a tissue the overall decay is a phasor that is the sum of the independent phasors of each fluorescence component:

$$G(\omega) = \sum_n f_n g_n(\omega) \quad (11)$$

$$S(\omega) = \sum_n f_n s_n(\omega) \quad (12)$$

Where f_n is the fractional contribution of each component characterized by the phasor coordinates g_n and s_n. Two molecular species with multi-exponential decay are identified by two specific points in the phasor plot inside the semicircle. All possible weighting of the two molecular species give phasors distributed along a straight line joining the phasors of the two species. In the case of three molecular species, all the possible combinations are contained in a triangle where the vertices correspond to the phasor of the pure species. The phasor plot of an N-component mixture will be contained in a polygon with N-vertices located in the position of the phasor of each contributing component. The calculation of the fractional intensities f_n of different fluorescence components that contribute to the signal is performed by a linear estimation on the system described by equation (11) and (12) by graphically resolving the sum of phasors.

4. CONCLUSIONS

Here we show that the phasor approach to FLIM can map stem cell and cancer cell metabolism in label-free cells and living tissues. This method provides a metabolic fingerprint of cells, mitochondria, cytoplasm and nucleus. Phasor approach is a “fit-free” method that requires no assumption or a priori knowledge on the biological system, such as its biochemical content. We separate and identify cells and tissue components by cluster analysis, i.e., detecting clusters of pixel values in specific regions in the phasor plot (Fig.1). We determine the phasor location of some relevant endogenous fluorophores, i.e. collagen, free and bound NADH, FAD, retinol, retinoic acid and porphyrin. The phasor location of every molecular species in the histogram is uniquely determined by their fluorescence decay. The phasor fingerprint of chemical species reduces the importance of knowing the exact lifetime

distribution of fluorophores decay and allows interpreting FLIM images directly in terms of chemical species. We measure and map the relative concentration of free and bound NADH in cells and living tissues. We calculate the FLIM phasor value of the cells, mitochondria, cytoplasm and nuclei.

The phasor approach in tissues is a promising tool in biology, biophotonics and biomedical research to track *in vivo* metabolic changes that are associated with stem cell differentiation, cell carcinogenesis and apoptosis. It could provide important insight into the signaling pathways and regulatory networks, which are involved in cell self-renewal differentiation and oncogenesis in a variety of tissue and organs. The phasor approach to FLIM would also be helpful to monitor cell metabolism and at the same time characterize the three-dimensional microenvironment of tissues by detecting extracellular matrix remodeling and molecular gradients. The ability to observe and isolate noninvasively cancer cells and stem cells based on their metabolic rate in living tissues has important implications for early diagnosis and new therapeutic strategies. The detection of malignant transformation of progenitor cells, aberrant differentiation of cancer cells can be performed in *vivo*. Label-free discrimination between self-renewal and differentiation by phasor approach to FLIM would be suitable to non-invasively monitor embryonic stem cells and to design new approaches to reprogram somatic cells to a pluripotent stem cell fate. The phasor approach to FLIM could be of interests to label-free cell sorting and high throughput screening for drug discovery, cell replacement therapies and tissue engineering.

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