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Real-time Analysis of Metabolic Activity Within *Lactobacillus acidophilus* by Phasor Fluorescence Lifetime Imaging Microscopy of NADH

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Abstract Nicotinamide adenine dinucleotide (NADH) is an endogenous fluorescent molecule commonly used as a metabolic biomarker. Fluorescence lifetime imaging microscopy (FLIM) is a method in which the fluorescence decay is measured at each pixel of an image. While the fluorescence spectrum of free and protein-bound NADH is very similar, free and protein-bound NADH display very different decay profiles. Therefore, FLIM can provide a way to distinguish free/bound NADH at the level of single bacteria within biological samples. The phasor technique is a graphical method to analyse the entire image and to produce a histogram of pixels with different decay profile. In this study, NADH fluorescence decay profiles within *Lactobacillus acidophilus* samples treated using different protocols indicated discernible variations. Clear distinctions between fluorescence decay profiles of NADH in samples of artificially heightened metabolic activity in comparison to those of samples lacking an accessible carbon source were obtained.

Introduction

Bacteria inhabit many environs that are subject to massive, and often rapid, fluctuations in environmental conditions. In order to adapt to these variations, bacteria rapidly adjust their proteomic complement and metabolic activity [1].

The fluorescence of reduced nicotinamide adenine dinucleotide (NADH), an important coenzyme of energy metabolism, has been used as a marker of bacterial metabolic activity for many years [2, 3]. While there are a number of endogenous fluorescent molecules within bacterial physiology, the contribution of NADH is considered to be the most significant for excitation in the near ultraviolet region [4].

Fluorescence lifetime imaging microscopy (FLIM) has been used to provide a real-time assessment of metabolic activity by visualizing the relative amount of free and protein-bound NADH in biological samples, independently of fluorescence intensity [5]. While the fluorescence spectrum of free and bound NADH is very similar, their lifetime differs significantly, of 0.4 ns in the protein-free state to 3.2 ns in the protein-bound state [6].

Since its inception, FLIM analysis has been almost exclusively focused on eukaryotic cells. However, the interaction of the obligate intracellular bacterium *Chlamydia trachomatis* and its host cell has been examined using NADH FLIM. In vitro evidence was found that bacteria involved in persistent chlamydial infection are able to be distinguished from cells capable of causing symptomatic outbreaks with this analysis [7]. In the aforementioned study, analysis of FLIM data was hindered by the lack of a clear method to distinguish between the NADH lifetimes of bacterial cells and the mammalian cytoplasm.

The phasor technique applied to FLIM provides a straight forward method to analyse lifetimes within an image at a pixel level [5, 8]. The phasor approach consists of performing a transformation of the decay profile in Fourier components. These components (sine and cosine transforms) are represented in a polar plot. Each pixel of an

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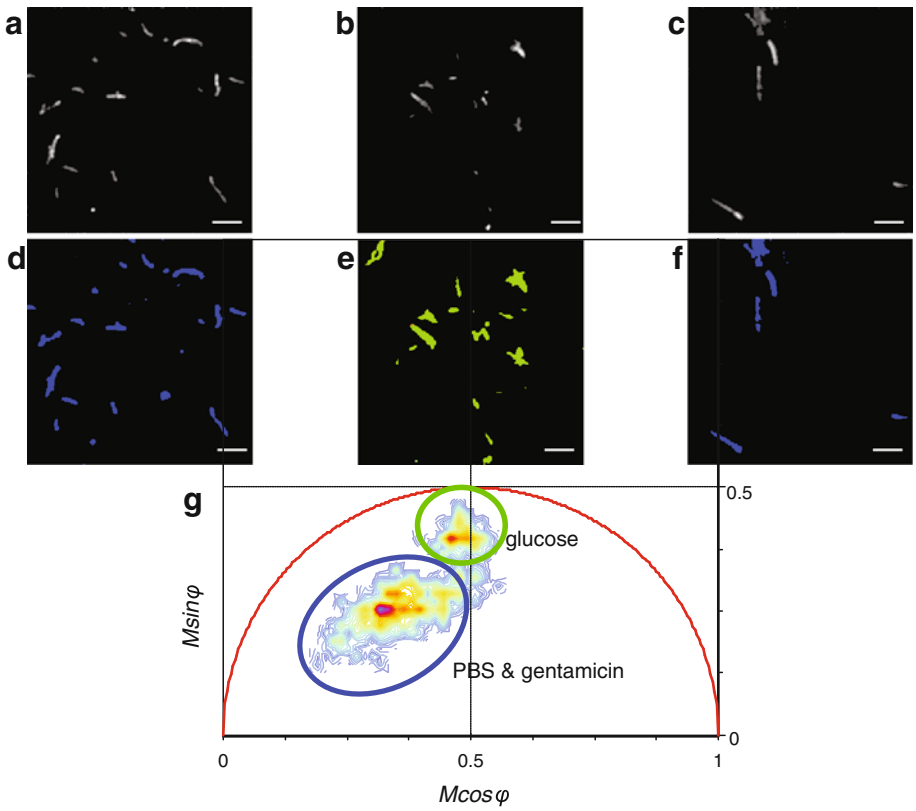

Fig. 1 Intensity images of *L. acidophilus* with **a** control, **b** glucose and **c** gentamicin treatments. Images (**d**–**f**) are intensity images where lifetimes have been colour coded according to selections in the **g** *phasor plot*. In the *phasor plot*, the lifetimes exhibited by glucose treatment (*green circle* at the apex of the graph) and the combined lifetimes exhibited by PBS and gentamicin treatment (larger *blue circle*). *Line* colour within *phasor plot* (from *blue* to *purple*) indicates the frequency of lifetime occurrence. *Scale bar* indicates 5 μm (Color figure online)

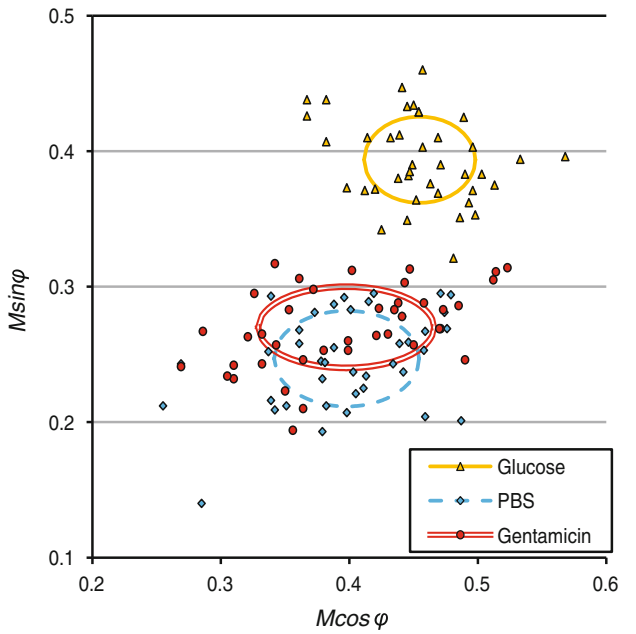

Fig. 2 *Scatter plot* of phasor lifetimes exhibited in random positions within bacteria of separate treatments (large *circles* indicate SD surrounding mean) clearly highlight the distinction in phasor positioning of NADH fluorescence lifetimes in glucose supplemented and carbon-source absent treatments

image has a corresponding point in the phasor plot and, vice versa, each point in the phasor plot corresponds to a pixel in the image. Therefore, if a part of an image, for example the cytoplasm, has a different ratio of free to bound NADH, all points in the cytoplasm will cluster in a region of the phasor plot [9]. A simple inspection of the phasor plot could reveal regions of different decay profiles. Phasor analysis has been used to discover real-time variations in NADH free/bound ratio distribution patterns within mammalian cells coincidental with transcriptional changes [9]. Phasor scatter plots, as introduced by Stringari et al. [5], provide a direct comparison of phasor positioning of selected regions and/or samples independent of the occurrence frequency of those lifetimes. In particular, Stringari et al. [10] established a metabolic index which illustrates a relationship between the position of the pixels in the phasor plot and the metabolic activity of cells.

In this study, the phasor approach is used to analyse NADH fluorescence lifetimes within *Lactobacillus acidophilus* to investigate distinctions between metabolic states.

Materials and Methods

The strain used in this experiment was *L. acidophilus* NCFM isolated from Inner Health Plus capsules (Ethical Nutrients) maintained on de Man, Rogosa, Sharpe agar (Oxoid). All biofilm examined were grown using brain

heart infusion broth (Oxoid) in glass bottom dishes (MatTek, Ashland, USA) incubated for 36 h at 37 °C.

Two hours prior to microscopic examination, biofilm were washed four times with 0.22-μm filter-sterilized phosphate-buffered saline (PBS). Following the washing procedure, the biofilm was subjected to three treatments in situ, (a) PBS (control), (b) PBS and glucose (5 % w/v) or (c) PBS and gentamicin (60 μg/ml).

An SP2 confocal microscope (Leica) was used for the acquisition of all FLIM data. The microscope was coupled to a titanium:sapphire Tsunami multi-photon laser (Spectra-Physics, Mountain View, CA) and a time-correlated single photon counting system from Becker & Hickl single photon counting module was synchronized with the microscope and pulse amplifier. For all FLIM acquisitions, a 63×/1.40 W CORR HCX PL APO objective was used. The scan speed was set to 400 Hz with an excitation wavelength of 740 nm (5 % laser power). Fluorescence was detected in a bandwidth of 430–620 nm. A fluorescein solution (50 mM) in 0.1 M NaOH was used for lifetime calibration.

Phasor technique was applied to FLIM acquired data using SimFCS software (Laboratory for Fluorescence Dynamics, University of California, Irvine). Phasor scatter plot data were obtained using a 0.0025 radius cursor (approximately 1 pixel) to select random positions within bacteria visualized.

Results and Discussion

Figure 1a–c shows typical fluorescence images obtained following subtraction of background fluorescence of weak intensity. The phasor plot (Fig. 1g) of the three treatments exhibit similar distributions for PBS and gentamicin treatments, while phasor position of NADH lifetimes in samples supplemented with glucose are closer to the apex of the universal circle. The frequency of lifetimes seen within the phasor plot (Fig. 1g) exhibit clustered regions towards the centre of the distributions. These distinctions correspond to those found in the scatter plot (Fig. 2).

The difference in phasor positioning of NADH lifetimes found in glucose-treated samples, and those found in PBS and gentamicin treatments (Fig. 2) indicates a significant shift in the environment of the molecule. As protein binding presents the most significant impact on NADH fluorescence lifetimes in biological samples [5], it could be considered that this reflects a change in concentration of the proteins which are interacting with NADH.

In previous studies with mammalian cells [9], the phasor position of NADH lifetimes was similar to those found in *L. acidophilus* samples supplemented with glucose. The change in NADH phasor lifetimes within PBS and gentamicin treatments reflects an environment experienced by NADH that is not frequently found in mammalian cells.

This study highlights the potential of the phasor–FLIM of NADH to provide a real-time in vivo method of distinguishing between bacterial populations of varied metabolic activity.

Further application of this technique to natural bacterial biofilm could be used to visualize the distribution of metabolic activity. The analysis of these distributions may yield to the detection of patterns which will provide insights into biofilm structure and function.

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