

Technical Note

Metabolic state profiling of organoids with FLIM

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Introduction

Organoids are three-dimensional assemblies of culture cells having similar functional characteristics as the complete organs in relation to specific stimuli or conditions. Their importance and influence in modern biological research have been highlighted by their designation as “Method of the year for 2017” by Nature Methods.

In this short technical note we demonstrate the possibility of identifying distinct metabolic profiles of organoids by Fluorescence Lifetime Imaging (FLIM) of autofluorescence arising from various states of bound and unbound NAD(H).

Sample Preparation

The experiments were conducted on mouse gastruloids, embryonic organoids formed by aggregation of mouse embryonic stem cells that mimic the formation of early embryonic tissues [1]. Gastruloids were prepared according to a previously developed protocol involving embryonic stem cell aggregation in the presence of Fgf and Activin and Wnt pathway activation as described previously [2]. Cells were aggregated at 0 hours in U-bottom low-adhesion culture plates and treated with the Wnt activator Chiron between 48 and 72 hours to induce cell differentiation. Organoids were generated using two different initial cell numbers (100 or 300), which influence organoid size and potentially establish metabolic gradients between the outer and inner cell layers. This is motivated by recent studies [3,4], which, based on metabolic interventions, suggest a mechanistic link between signaling activity (e.g. Wnt signaling) and metabolism (e.g. glycolysis vs. oxidative phosphorylation).

To investigate different stages and treatment effects, the following conditions were investigated:

- Time points: 72 hours (early) and 96 hours (late maturation)
- Treatment: untreated and Chiron-treated (+Chi / –Chi)
- Cell line: E14 wild-type (no transgenic fluorescent protein expression)

Instrumentation

Fluorescence lifetime images were acquired using the [Luminosa](#) single-photon counting laser scanning confocal microscope from PicoQuant. [Luminosa](#) combines intuitive workflows, online image previews for real-time quality assessment, and advanced and fast GPU-based analysis routines. The microscope system is fully motorized, allowing for quick auto-alignment and power calibration. Measurements were performed using a 60× water immersion objective with a numerical aperture (NA) of 1.2.

Two-photon excitation was performed with the TOPTICA fs-Laser Add-On for Luminosa, incorporating the [FemtoFiber® ultra 780](#) laser system. This system is based on a passive mode-locked Erbium-doped fiber oscillator, followed by a high-power fiber amplifier and second harmonic generation (SHG) unit. It also includes a group delay dispersion (GDD) pre-compensation module, used for compensation for the Luminosa microscope's optical beam path. The pulses are centered at 780 nm, with a pulse duration of approximately 140 fs and a repetition rate of 50 MHz.

Measurements were conducted under physiological conditions using the [TOKAI HIT Stage Top Incubator®](#) at 37°C and an STXG controller for 5% CO₂ + 95% air mixture supply to the chamber over a period of at least 96 hours.

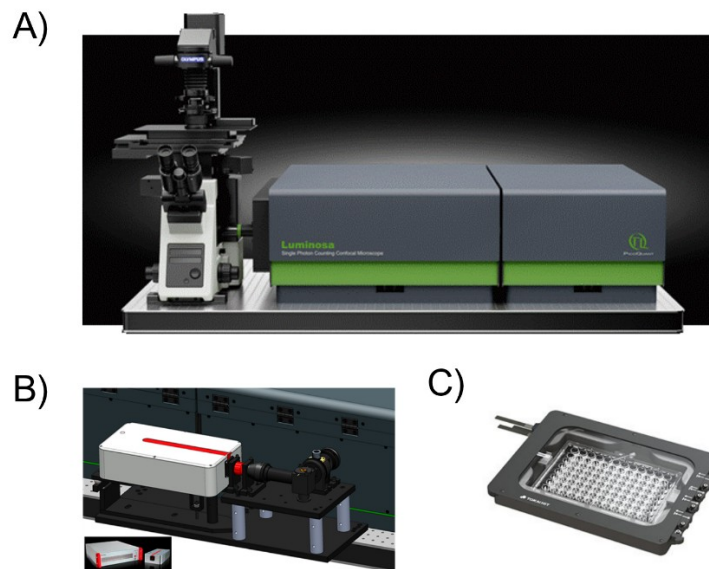


Fig. 1: A) Luminosa single photon counting confocal microscope. B) Coupling of the [FemtoFiber® ultra 780](#) from an additional excitation laser port. C) The [TOKAI HIT Stage Top Incubator®](#).

Experiments

All measurements were performed with average laser power of up to 20 mW at the sample plane. The Toptica fs-laser system power can be controlled from the Luminosa software. The main dichroic used was a shortpass 670, the pinhole was open to 5 AU (= 476 μ m).

The detector used was a PMA Hybrid (PMA Hybrid Series -40). For NADH imaging a bandpass filter 483/40 was used.

To minimize photo-induced damage of the sample due to high laser powers, most images were recorded with short pixel dwell times of around 10 μs and multiple frames between 80 and 130, with a pixel size between 190 and 310 nm over a typical field of view of 50–80 μm .

The images were further analyzed with the [NovaFLIM](#) software, which allows to seamlessly apply various analysis schemes: Phasor Plots, FastLifetime contrast, multi-exponential fitting and pattern matching analysis.

Imaging Results

Two-photon excitation enables deeper penetration depth due to longer excitation wavelengths. Fig. 2 presents an organoid scan in around 55 μm depth inside the organoid. The 780 nm excitation wavelength provides the benefits of lower photon-count losses as well as reduced imaging resolution degradation at substantial imaging depths. This image was recorded with 1-frame scan, pixel dwell time of 1 ms, 189 nm pixel size, 97 x 97 μm field-of-view.

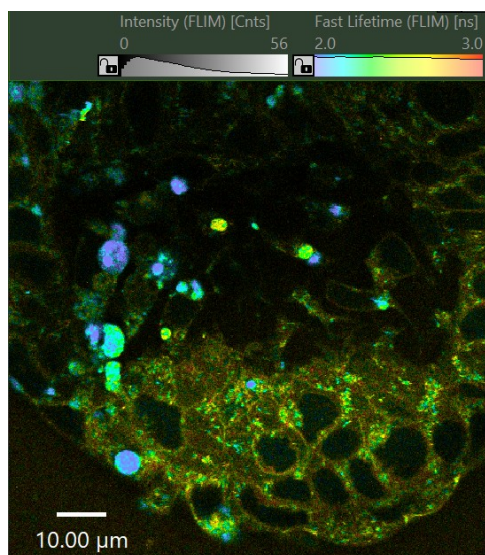
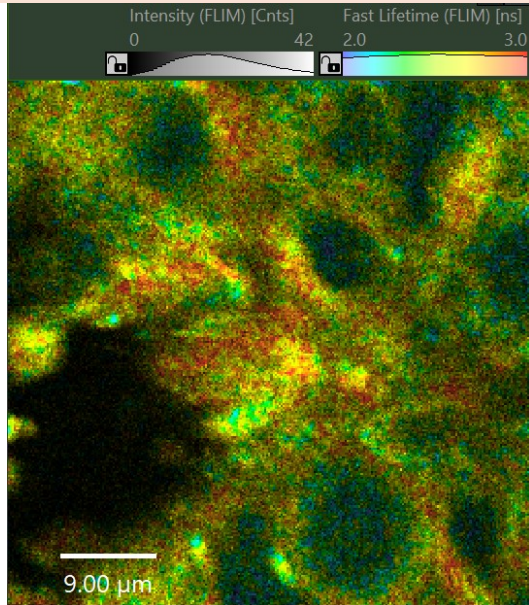


Fig. 2 FastLifetime image of organoid cross-section scan (after over 168 hours of maturation without shaking). The imaging plane was around 55 μm inside the organoid.

In this short tech note we focused on identifying changes in the metabolic profiles of the organoids simply by observing the phasor plot histograms. Fig. 3 compares the phasor plot of a scan of organoids from the E14 untreated cell line after around 72 hours of maturation (a) and of Chi-treated organoids after around 96 hours of maturation (b). The plots indicate that lifetime is reduced for treated organoids with longer maturation time in comparison to untreated organoids with shorter maturation time. This trend is also visible in the corresponding FastLifetime images.

E14 wild type

Non-treated , 72hours



Chi-treated , 96hours

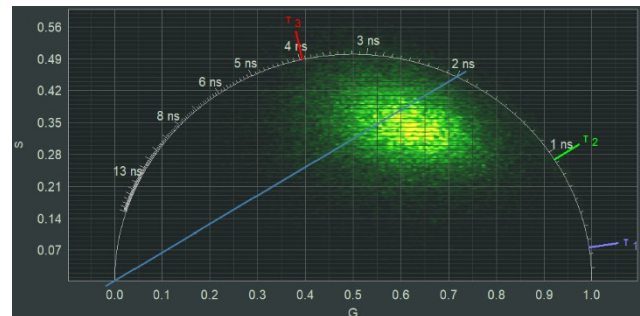
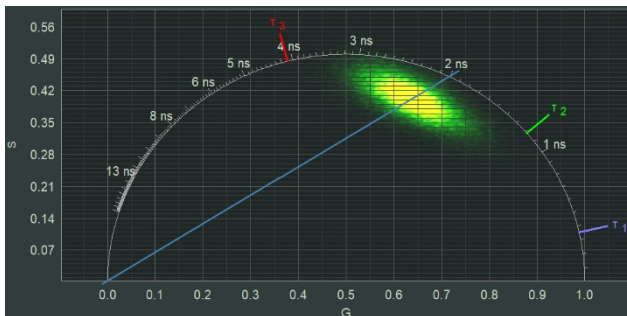
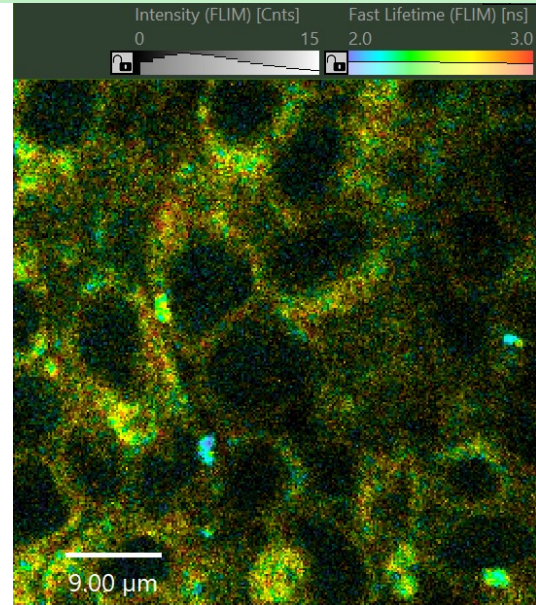


Fig. 3: Organoids from E14 cell line and initial cell number of 300. Figures as displayed in NovaFLIM software. a) FastLifetime image and phasor plot of untreated organoids (i.e. without Wnt activation) after around 72 hours of maturation. b) FastLifetime image and phasor plot of organoids treated with the Wnt activator after around 96 hours of maturation. Diagonal line added to help identify phasor population shifts towards shorter lifetimes.

Summary and Outlook

We have shown that metabolic profiles of growing organoids can be characterised via FLIM in Luminosa microscope. The integration of the [FemtoFiber® ultra 780](#) laser offers an extremely robust, reliable and cost effective option for 2-photon excitation of NAD(H) , whereas the [TOKAI HIT Stage Top Incubator®](#) allowed the organoids to grow without noticeable growth defects for up to almost 200 hours on the microscope stage. We envision that the streamlined acquisition and analysis workflow offered by Luminosa will be a huge step forward in metabolic profiling of organoids and the development of various organoid arrays as screening platforms.

References

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