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Label-Free Flow Cytometric Measurements of Cellular Metabolic Factors for Detection of Cancer Cells

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BACKGROUND

Kinetic River has leveraged the use of fluorescence lifetime flow cytometry techniques to develop the *Tiber*, an instrument capable of using measurements of fluorescence decay after pulsed excitation (**Figure 1**) to distinguish between normal and cancerous cells. In contrast to conventional flow cytometry, which uses a continuous light source to generate a single fluorescence peak per event, measurement of lifetime under flow conditions requires the use of a modulated light source and generates hundreds of peaks per event (**Figure 2**).

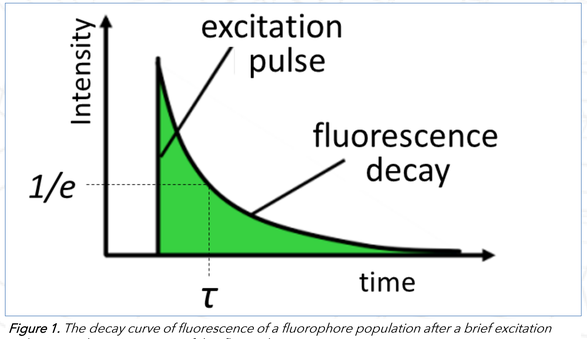

Figure 1. *The decay curve of fluorescence of a fluorophore population after a brief excitation pulse is an inherent property of that fluorophore.*

The *Tiber* takes advantage of the well-known Warburg Effect, whereby cancerous cells use glycolysis as their favored metabolic pathway, whereas normal cells use oxidative phosphorylation. Two ubiquitous cellular coenzymes, NADH (the reduced form of nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide) have distinct biochemical differences in these two different metabolic states, based on whether they are predominantly free or protein-bound. Both coenzymes exhibit strong autofluorescence, particularly under UV and violet excitation. More importantly, they each display distinct fluorescence lifetime characteristics depending on which state they are in (**Table 1**). Results from fluorescence-lifetime imaging microscopy (FLIM) confirm this (**Figure 3**). Kinetic River's *Tiber* instrument has translated this technique to the high-throughput realm of flow cytometry, taking advantage of this phenomenon to create a simple, generalized method to distinguish cancerous cells from non-cancerous ones. Relying on autofluorescence of endogenous molecules, this method is label-free, not requiring the addition of exogenous fluorophores.

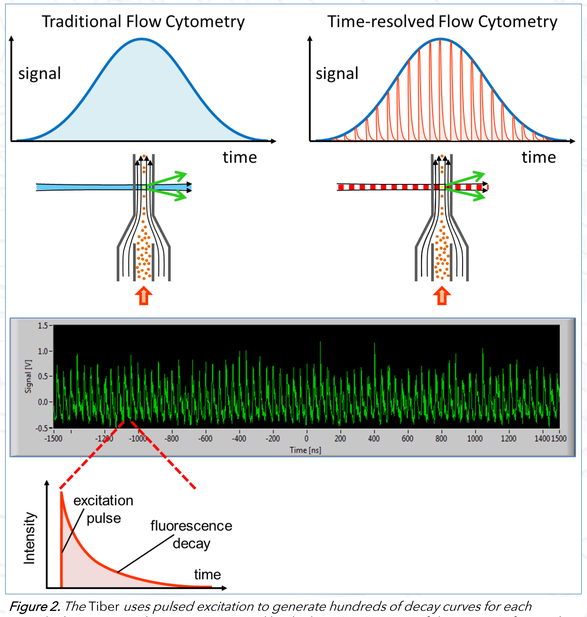

Figure 2. *The* Tiber *uses pulsed excitation to generate hundreds of decay curves for each particle that traverses the area interrogated by the laser (top). A train of decay curves from a dye solution (bottom) shows the repeated pattern of excitation and fluorescence decay.*

METHODS

The system uses a 375-nm pulsed laser source (Omicron Laserage) for excitation of NADH and FAD autofluorescence. The optical design is based on the use of emissions bands that span from 380 nm to 525 nm. The emitted light is collected by two fluorescent detectors (Hamamatsu PMTs) and two detectors to collect the scattered light (FSC and SSC detectors). The system is supported by *Shasta*, our custom-designed and -built fluidic system (see Poster #261 – "A Highly Customizable Fluidics Control Module for Flow Cytometry). The signals are processed using our proprietary time-domain waveform analysis technology.

Cell type	Metabolic Pathway	α free (short τ value)	α bound (long τ value)
Normal	oxidative phos. (in mitochondria)	↓	↑
Cancerous	aerobic glycolysis (cytosolic)	↑	↓

α_{free} and α_{bound} refer to the fraction of NADH that is found free or protein-bound

Free NADH lifetime, τ_{free}, is SHORT, <0.5 nsec

Protein-bound NADH lifetime, τ_{bound}, is LONG, >1.5 nsec

Table 1. *Whereas normal cells utilize oxidative phosphorylation, cancerous cells utilize the less efficient aerobic glycolysis. This metabolic shift seen in cancerous cells–the so-called Warburg Effect–results in a shift in the proportion of cellular NADH and that is protein-bound versus free. Free NADH has a distinctly different fluorescence lifetime than when protein-bound. These differences can be easily measured by the* Tiber *instrument.*

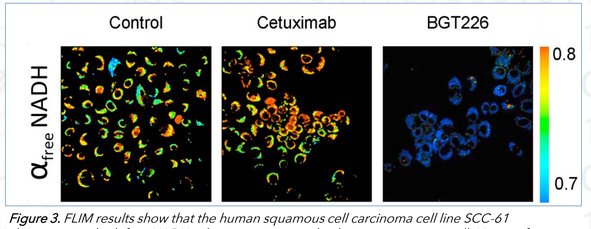

Figure 3. *FLIM results show that the human squamous cell carcinoma cell line SCC-61 demonstrates high free NADH values, consistent with it being a cancerous cell. No significant changes in this value are observed after treatment with the anti-EGFR antibody cetuximab. However, early metabolic changes were detected in response to treatment with BGT226, a PI3K/mTOR inhibitor. The method may be useful in detecting early responses to treatment and guiding therapy decisions for cancer patients. From Shah* et al. *(2014) PLoS ONE 9(3): e90746*

RESULTS

We have designed and built the *Tiber* system. Characterization was performed using Spherotech beads to assess alignment, CVs, dynamic range and sensitivity of each channel. Spectral testing was performed using fluorophores in solution. Key preliminary results demonstrate that the *Tiber* is able to resolve two distinct lifetimes of NADH, corresponding to the free and the bound forms (**Figure 4**). Work continues to characterize measurements of free and bound FAD, as well as of NADH and FAD in combination, followed by biological validation in a relevant cell-based assay.

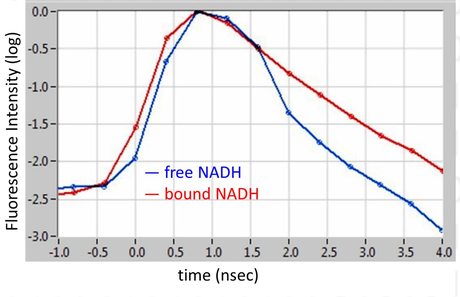

Figure 4. *Fluorescence lifetime measurements of NADH both free (blue, short lifetime) and bound to lactate dehydrogenase (red, long lifetime) taken on the* Tiber *yielded consistent and measurable differences between the two species.*

CONCLUSION

The *Tiber* system design leverages our previous work on integrating time-resolved fluorescence measurements in flow cytometry (see Poster #269 - "Automated Elimination of Autofluorescence Interference in Flow Cytometry", and Poster #292 - "Compensation-Free Flow Cytometry"). The instrument is aimed at providing a unique high-throughput, label-free method for cancer cell discrimination at the single cell-level. Future work will build on the feasibility we have demonstrated here by validating the system performance using cell-based systems with both cancerous and non-cancerous cells.

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The Tiber *and* Shasta, *or use thereof, may be covered in whole or in part by patents in the U.S. and other jurisdictions. A current list of applicable patents can be found at https://www.kineticriver.com/kinetic-river-corp-patents.*