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# A high content imaging flow cytometry approach to study mitochondria in T cells: MitoTracker Green FM dye concentration optimization

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ABSTRACT

Mitochondria, the powerhouse of the cell, are known to remodel their membrane structures through the process of fusion or fission. Studies have indicated that T cells adopt different energy metabolic phenotypes, namely oxidative phosphorylation and glycolysis depending on whether they are naïve, effector and memory T cells. It has recently been shown that changes in mitochondrial morphology dictate T cell fate via regulation of their metabolism. Our keen interest in T cell function and metabolism led us to explore and establish a method to study mitochondria in live T cells through a novel high content approach called Imaging Flow Cytometry (IFC).

The focus of our current study was on developing a protocol to standardize the concentration of MitoTracker Green FM dye to observe mitochondria in live T cells using IFC. We began the study by using widefield microscopy to confirm the localisation of MitoTracker Green FM labelled mitochondria in live T cells. This was followed by testing various concentrations of the dye to achieve a similar labelling pattern using IFC while eliminating false positive or negative staining. The optimization of the method used to label the mitochondria by IFC for analysis included standardisation of a number of important parameters such as dye concentration, voltage, fluorescence intensity values for acquisition and processing. IFC could potentially be a powerful method to study T cells in a relatively high throughput manner.



## 1. Introduction

### 1.1. T cells and their functional subsets

T cells are an important subset of the adaptive branch of the immune system. They arise from lymphoid precursors in the bone marrow, which travel through the blood and enter the thymus [1]. The thymus, classified as a primary lymphoid organ, is the organ where T cells develop, and become educated and selected to express a useful antigen-recognition repertoire. After selection and final differentiation, they migrate to the periphery. This selection is important to make the T cells capable of mounting an immune response against non-self-antigen fragments presented by self Major Histocompatibility Complex (MHC) proteins, whereas they must remain non-responsive to self-antigens. In this way, they refrain from the development of autoimmunity. Following this selection, T cells migrate from the thymus to the periphery [2,3].

The three known major functional subsets of T cells are Naïve ($T_N$), Effector ($T_E$) and Memory ($T_M$). When a $T_N$ cell residing in secondary lymphoid organs such as lymph nodes, encounters its cognate antigen and receives an adequate amount of co-stimulatory signal, it transforms into an activated T cell. The $T_N$ undergoes extensive proliferation termed as "clonal expansion", ultimately differentiating into $T_E$ cells. Upon clearance of antigen or infection, almost the entire population of these short-lived $T_E$ cells is lost during the contraction phase of the response. Another small subset of T cells derived from $T_N$ cells differentiates into long lived $T_M$ cells, which helps in mediating a quicker and enhanced immune response upon subsequent exposure to the same antigen [4,5].

### 1.2. Immunity and metabolism: OXPHOS/glycolysis in T cells

Recently a multitude of studies point towards a connection between immunity and metabolism. There is an increasing evidence that T cells are able to perform their respective functions only by virtue of their variable, function-specific energy requirements [6]. The two known energy generation pathways adopted

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by T cells are Oxidative phosphorylation (OXPHOS) and aerobic glycolysis [7].

It has been shown that quiescent T cells such as $T_N$ and $T_M$ cells primarily use OXPHOS, a highly efficient (generating 36 ATP molecules from one glucose molecule) catabolic pathway. In contrast, $T_E$ cells adopt aerobic glycolysis, a less efficient (generating 2 ATP molecules from one glucose molecule) anabolic pathway as their key energy pathway [7]. This switch to aerobic glycolysis, also known as the Warburg effect, is a characteristic feature of highly proliferative tumor cells [8]. Thus, modulation of the T cells' metabolic pathways and needs facilitates their ability to perform the functions of the particular subset of T cells [9].

### 1.3. Mitochondria and immunity: Powerhouse of T cells

Studies have shown that mitochondria, double membranous organelles, are critical regulators of immunity and play essential roles in T cells [10]. Mitochondria are known as the powerhouse of the cell because it is the site where OXPHOS occurs. In T cells, as in other cells, energy substrates like glucose, fatty acids and glutamine get converted into products which participate in the tricarboxylic acid (TCA) cycle localised in the mitochondrial matrix. This cycle generates two electron carriers namely NADH and $FADH_2$, which serve as electron donors for the electron transport chain (ETC) comprising of four complexes present in the inner mitochondrial membrane. The sequential transfer of electrons builds up a proton gradient due to simultaneous pumping of protons from the matrix into the inter membrane space. The proton gradient is thus utilised to fuel the production of ATP from ADP and inorganic phosphate [11,12]. Moreover, mitochondria aid in antigen-induced T cell activation via generation of reactive oxygen species (ROS) [13], regulation of $T_M$ metabolism via fatty acid synthesis [14], T cell aging, autoimmunity [12], maintenance of calcium homeostasis [15], regulation of programmed cell death [16] and anti-viral immunity [17].

### 1.4. Mitochondrial morphology: Indicator of OXPHOS/glycolysis in T cells

Mitochondria possess the intrinsic property to continuously change shape by undergoing fission or fusion. This is tightly linked to their ability to perform their functions [18]. A recent study indicates that changes in the mitochondrial morphology dictate T cell fate via regulation of their metabolism [19]. Naïve T cells show a "fused" morphology with relatively low density, but $T_M$ cells undergo mitochondrial fusion resulting in mitochondria with elongated tubular morphology facilitating efficient OXPHOS [19]. On the other hand, $T_E$ cells undergo mitochondrial fission and have punctate mitochondrial morphology resulting in inefficient OXPHOS. The punctate mitochondria adopt glycolysis as their major pathway of energy production [19].

### 1.5. MitoTracker probes

The advent of fluorescent dyes has revolutionised the study of various organelles under different experimental conditions, as these dyes are able to directly label the organelles allowing them to be visualised under a microscope. Fluorescent dyes used to label mitochondria include the MitoTracker probes, which are available conjugated to a wide variety of fluorochromes. Although they have distinct properties that allow the study of the functions, morphology and location of mitochondria, they share a common staining principle [20,21].

MitoTracker probes contain a mildly thiol-reactive chemical group which mediates selective binding to mitochondria [22,23]. Another advantage of these probes is the ease with which mitochondrial staining is achieved, as they can diffuse non-energetically across the plasma membrane.

### 1.6. MitoTracker Green FM dye

MitoTracker Green FM dye has bright green fluorescence and three specific advantages which make it an appropriate choice to visualize mitochondria in live T cells [24]. Firstly, it is a highly mitochondria-selective dye and a suitable chemical tool to study the mitochondrial mass due to its insensitivity to changes in mitochondrial membrane potential. [25]. Due to its insensitivity to membrane potential, this dye can be used to label the total mitochondrial pool (both healthy and unhealthy mitochondria). Secondly, it can produce a bright mitochondria specific signal even at low concentrations due to its photo-stability. Lastly, the bright signal and selectivity for mitochondria can be attributed to the ability of the dye to fluoresce only in the lipid environment found within the mitochondria and not in aqueous solution, which greatly decreases the background, making it an ideal choice for imaging purposes [24].

It is important to note that although our study discusses the use of only MitoTracker Green FM to visualize mitochondria in T cells, other bulk mitochondrial stains like MitoTracker Red and Deep Red can also be used in this procedure. Both MitoTracker Red and Deep Red are used to label live mitochondria and this labelling is dependent on membrane potential (unlike labelling using MitoTracker Green FM) [22]. Therefore, only the mitochondria which are healthy and thus able to maintain the membrane polarity to generate ATP during oxidative phosphorylation are labelled. An interesting set of experiments using this protocol could be performed by combining MitoTracker Red/MitoTracker Deep Red and MitoTracker Green FM to assess the different physiological states of mitochondria versus total mitochondria in T cells subjected to various experimental conditions [26,27].

### 1.7. High content imaging flow cytometry (Amnis): A novel approach to study T cell mitochondria

Conventionally, microscopy has been used for imaging single cells or small populations of cells while flow cytometry has been used for multi-wavelength fluorescence analysis of millions of cells. Recently, the development of a novel technique termed Imaging Flow cytometry (IFC) has transformed the world of high throughput imaging [28]. IFC, as its name suggests, is an amalgamation of microscopy and flow cytometry which allows the user to obtain images of thousands of cells in a single-cell suspension.

We were keen to utilise this novel approach to study the mitochondria in T cells for the following reasons. IFC analysis of mitochondria in T cells can be performed in an unbiased, high content manner. In contrast, microscopy can potentially be limited by the number of cells that can be analysed. IFC allows us to detect the fluorophore-tagged molecule on the cell surface as well as internal to the cell, which cannot be achieved by flow cytometry [29]. Moreover, it allows automated computational analysis of vast amounts of data (typically, thousands of images) using >85 varied morphological characteristics that can be user defined and can be applied to both brightfield and fluorescent channels. It also provides the user a unique ability to design a combination of masks or formulas to study the parameters of their choice [29].

In this article, we discuss the methods used to optimize the concentration of MitoTracker Green FM dye to observe mitochondria in live T cells using IFC. First, the MitoTracker Green FM labelled mitochondria in live T cells were visualised by widefield microscopy to confirm their localisation. Next, the following two parameters were strictly monitored to optimize the IFC protocol: a) that the mitochondrial localisation from the IFC was similar to what