Miltenyi Biotec

MACSQuant® Analyzer X

Calcium flux assay in mixed populations using kinetic flow cytometry

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Key Information

- Kinetic flow cytometry is an ideal approach to run a calcium flux assay on a mixed cell population.
- Cayman's reagents thapsigargin and fluo-3 AM can be used with Miltenyi Biotec's recombinant antibodies and MACSQuant X Flow Cytometer to test calcium flux in multiple cell types simultaneously.
- This simple approach can be applied to many complex cell mixes, from peripheral blood mononuclear cells (PBMCs) to transfected cells or dissociated tissues.

Introduction

Calcium is a tightly regulated and important second messenger molecule in many types of cells, notably immune cells. Calcium movement between cellular compartments and in and out of cells (calcium flux) is a highly complex process and has been studied extensively in isolated cell populations and cell lines.[1] A series of fluorescent dyes has been developed to specifically detect free calcium within cells. Traditional calcium flux assays have largely been limited to single cell types, as most have been performed using plate readers, which monitor fluorescence over time in a whole population. Two clear strengths of flow cytometry are the ability to label and differentiate subpopulations within a complex mixture of cells (such as PBMCs) and single-cell level data. However, the requirement of many flow cytometers to have the sample tube under pressure necessitates a break in data collection for stimulus addition. The MACSQuant X Flow Cytometer utilizes a peristaltic pump for continuous sample acquisition, making the addition of stimulus without pausing acquisition possible.

Thapsigargin is a well-studied inhibitor of the sarco/endoplasmic reticulum (ER) calcium ATPase (SERCA), which causes depletion of ER calcium stores. This depletion can cause a secondary activation of plasma membrane calcium channels, ultimately leading to ER stress, unfolded protein response, and cell death. Different cell types, however, regulate these processes in different ways, potentially resulting in altered calcium influx in different cell types within a complex population. In this study, we investigated calcium influx responses in peripheral blood leukocytes stained with recombinant antibodies to differentiate specific cell types.

Methods

Blood was drawn into heparinized tubes, and red blood cells (RBCs) were lysed using an ammonium-chloride-potassium (ACK) lysis buffer. Cells were pelleted, washed, and resuspended in assay buffer (HBSS without calcium and magnesium), and 1.0 x 10^6 cells were transferred to fluo-3 AM staining buffer (HBSS without calcium and magnesium, plus 0.04% pluronic F-127, 2.5 mM probenecid, and 2 µM fluo-3 AM) and incubated at 37°C for 30 minutes. Cells were pelleted by centrifugation at 250 × g for five minutes and resuspended in assay buffer with or without 1 µM of thapsigargin for 15 minutes, and with surface marker antibodies (table 1) for 10 minutes at room temperature. An unstained control, as well as compensation controls, was acquired on the MACSQuant X Flow Cytometer. For fully stained samples, 500 µL of sample was acquired on HIGH, and calcium chloride was added to 2.5 µM at 20 seconds of acquisition. The data thus obtained was analyzed using FlowLogic™ Software.

Results

Forward and side scatter were initially utilized to divide total leukocytes into granulocytes, which have high side scatter and consist mostly of neutrophils, and mononuclear cells (PBMCs), which are a mixed population of lymphocytes and innate cells. Surface marker staining was utilized to divide the PBMC population into innate cells (monocytes [$CD14^+$] and NK cells [$CD56^+$]), as well as lymphocytes (B cells [$CD19^+$] and T cells [$CD3^+$]), and the T cell population further into naïve [$CD45RO^-$] and memory [$CD45RO^+$]. The total population, and each of these subpopulations, was analyzed for calcium flux after addition of extracellular calcium to thapsigargin-treated cells (fig 1).

Upon initial examination of the total cell population, the calcium flux began at approximately 130 seconds into acquisition and was a highly variable response across all the cells. Qualitatively, B cells and NK cells showed the least dramatic calcium flux of all cell types, which began later (more than 150 seconds into acquisition) and reached lower maximum fluorescence than other cell types. On the other hand, neutrophils and monocytes showed clear and robust responses to calcium addition, with earlier (130 seconds), fast (complete within about 30 seconds), and strong (10–25 fold increases in mean fluorescence intensity) calcium influx into the cells.

Within the total $CD3^+$ T cell population, the response appeared mixed, with some T cells showing strong response to calcium, and others lagging. When the population was divided based on CD45RO expression (a marker of memory T cells), the response became clearer. While $CD45RO^-$ naïve T cells showed a heterogenous response to calcium addition, memory cells, which are primed to respond quickly to threats, showed a clear and robust response to calcium addition post-thapsigargin treatment. This suggests memory cells have a greater ability to quickly replace calcium when intracellular stores are emptied than their naïve counterparts.

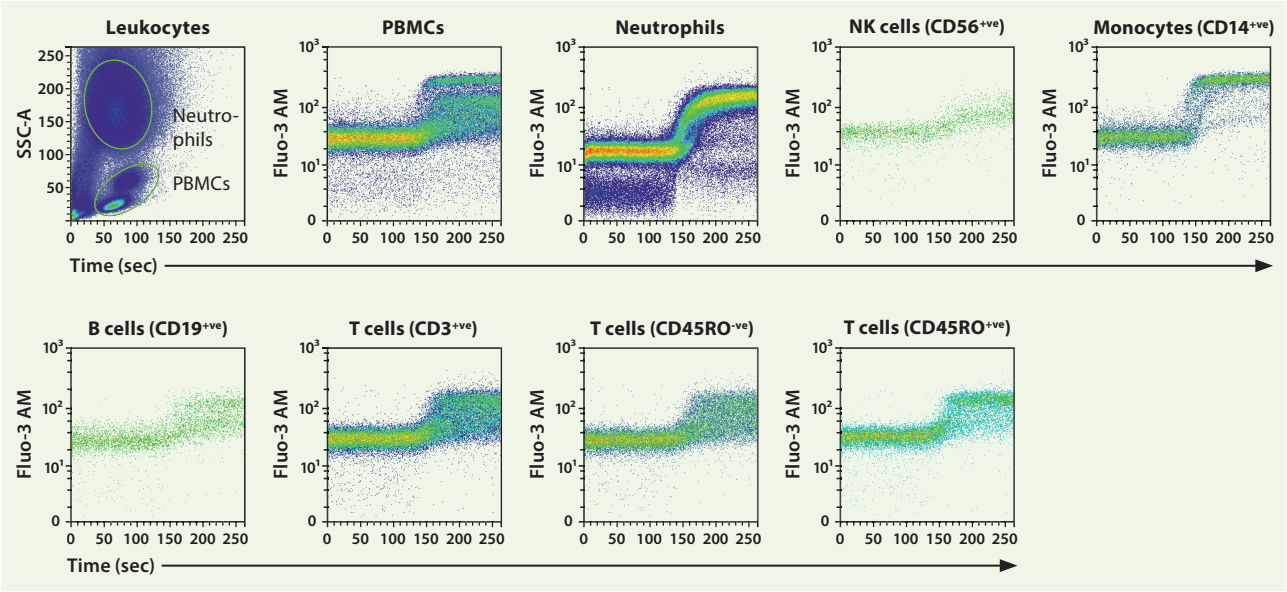

Figure 1: PBMC calcium flux by cell type. Total leukocytes were gated in FlowLogic™ by forward and side scatter into a granulocyte population (mostly neutrophils) and a PBMC population. The PBMCs were further segmented into NK cells, monocytes, B cells, and T cells (naïve and memory) based on surface marker expression. Each population was visualized for fluo-3 AM fluorescence over time.

Conclusion

As a signaling molecule, calcium plays a critical role in the activation of many different immune cell types, and thus is very tightly regulated by complex processes. To better understand these processes, methods must be available to investigate calcium flux in a multiplexed manner so as to allow the identification of subpopulations within a mixture of cells. We have shown here that, together with a well-characterized calcium dye, fluo-3 AM, Miltenyi Biotec's MACSQuant® X Flow Cytometer provides a very sensitive way to measure calcium flux in antibody-labeled PBMCs. This methodology is highly translatable to nearly any cell mixture and may even be able to examine calcium flux in very rare populations. We used thapsigargin to deplete intracellular calcium stores and stimulate calcium release-activated calcium channels, but any stimulus for calcium flux would be possible with this system. The continuous sample uptake of the MACSQuant X Flow Cytometer allows for uninterrupted detection of fluorescence changes in each sample, which is key to investigating the rapid changes inherent in the calcium flux system.

Reference

1. Lock, J.T., Parker, I., and Smith, I.F. A comparison of fluorescent Ca^{2+} indicators for imaging local Ca^{2+} signals in cultured cells. *Cell Calcium* **58(6)**, 638-648 (2015).

Products	Clone	Fluorochrome	Provider item no.
CD3	REA613	PE	Miltenyi Biotec 130-113-139
CD14	REA599	PE-Vio® 770	Miltenyi Biotec 130-110-521
CD19	REA675	APC-Vio 770	Miltenyi Biotec 130-113-643
CD45RO	REA611	VioBlue®	Miltenyi Biotec 130-119-620
CD56	REA196	APC	Miltenyi Biotec 130-113-310
REA control	REA293	APC	Miltenyi Biotec 130-113-434
REA control	REA293	VioBlue	Miltenyi Biotec 130-113-454
MACSQuant Analyzer X			Miltenyi Biotec 130-105-100
Fluo-3 AM			Cayman Chemical 14960
Thapsigargin			Cayman Chemical 10522
Probenecid			Cayman Chemical 14981

Table 1: Products used in this application.

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