Journal of Immunological Methods xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Imaging flow cytometry: A method for examining dynamic native FOXO1 localization in human lymphocytes

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ARTICLE INFO

Keywords:
FOXO1
Localization
Imaging flow cytometry
CD4
CD8
Human
Lymphocytes

ABSTRACT

While flow cytometry can reliably assess surface and intracellular marker expression within small cell populations, it does not provide any information on protein localization. Several key transcription factors (TF) downstream of lymphocyte surface receptors are regulated by nuclear versus cytoplasmic localization, and one such TF is Forkhead box O1 (FOXO1). FOXO1 integrates antigen-binding, co-receptor activation and metabolic signals in lymphocytes, leading to proliferation and differentiation. Importantly, the nuclear or cytoplasmic localization of FOXO1 is key for gene expression leading to different lymphocyte phenotypes. In effector lymphocytes (Teff), for example, lymphocyte receptor (TCR) signaling leads to an Akt-dependent phosphorylation of FOXO1. Phosphorylated FOXO1 is excluded from the nucleus, promoting proliferation and effector functions. In contrast, nuclear retention of FOXO1 is essential for early and late development of T and B cells and for the thymic development and stability of regulatory T cells. Given the critical role of FOXO1 localization as an indicator and determinant of function, quantification of FOXO1 cellular localization in human lymphocytes can help determine immune cell activation and activity in experimental and clinical scenarios. The standard method used to determine subcellular protein localization is the analysis of nuclear and cytoplasmic protein extracts by Western blotting (WB). However, available techniques, such as WB, are limited by a requirement for a large number of cells and inability to determine FOXO1 localization in individual cells or sub-populations. In contrast, a standardized method using an imaging flow cytometer (IFC) such as the Amnis Imagestream^X Mark II, would provide both qualitative, per-cell localization information, as well as quantitative data on gated sub-populations. To this end, we report the development and optimization of an IFC protocol to examine native FOXO1 localization in human lymphocytes. A human CD4+ lymphocyte line, HuT102, as well as primary human T cells, were assessed for dynamic FOXO1 localization after treatment with a lymphocyte receptor signaling mimic (PMA/Ionomycin). IFC nuclear translocation analysis permitted us to precisely quantify the alterations over time in nuclear and cytoplasmic localization of native FOXO1 on a per cell basis, including within specific, user-defined sub-populations of cells. For human lymphocytes, using IFC to assess and quantify dynamic FOXO1 localization allows the user to simultaneously study multiple lymphocyte subpopulations as well as to delineate differing effects of dynamic FOXO1 localization that may be lost when other available methods are used.

1. Introduction

To study lymphocyte activation in clinical samples, which often are restricted in size and quantity, it is necessary to gain the greatest amount of data possible from the fewest available cells (Pockley et al., 2015; Shapiro, 2003). Flow cytometry offers a distinct advantage for studying rare lymphocytes, using gating strategies to identify many different cell types. Multiple flow cytometry-based strategies for measuring the precise activation status of lymphocytes have been advanced, including cell surface activation markers and phospho-flow cytometry (Kaminski et al., 2012; Krutzik et al., 2004; Krutzik et al., 2011). However, flow cytometry is unable to provide concurrent information on protein localization, another key component of lymphocyte activation (Barteneva et al., 2012). This deficiency in standard flow cytometry precludes the thorough study of certain families of transcription factors whose localization influences their function

Abbreviations: TF, transcription factor; FOXO1, Forkhead box O1; WB, Western blotting; PMA/I, phorbol 12-myristate 13-acetate/ionomycin; Tregs, CD4+ regulatory T cells; Teff, CD4+ effector T cells; IFC, imaging flow cytometry; Akti, Akt inhibitor VIII; PBMCs, peripheral blood mononuclear cells

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https://doi.org/10.1016/j.jim.2018.01.001
Received 14 September 2017; Received in revised form 28 November 2017; Accepted 8 January 2018
0022-1759/ Published by Elsevier B.V.

(Vaquerizas et al., 2009). One such molecule that influences lymphocyte fate and function is Forkhead box O1 (FOXO1) (Ushmorov & Wirth, 2017; Wang et al., 2014).

FOXO1 plays a central role in regulating CD4+ lymphocyte differentiation and activation to mediate a balanced immune response via its effects on both regulatory lymphocytes and effector CD4+ lymphocytes (Hedrick et al., 2012). Nuclear FOXO1 acts as a TF that both negatively (e.g. *Sema4A*) and positively (e.g. *Foxp3* and *Klf2*) regulates gene expression (Ouyang et al., 2012) in both CD4+ regulatory T cells (Tregs) and CD4+ effector T cells (Teff) and at different stages of T cell development (Hedrick et al., 2012; Kerdiles et al., 2010). FOXO1 itself is regulated by lymphocyte signaling–when the PI3K/Akt pathway is activated downstream of lymphocyte receptor and co-stimulation, Akt phosphorylates FOXO1 and excludes it from the nucleus, preventing it from acting as a TF and allowing for potential ubiquitination and degradation (Wang et al., 2014). CD8+ lymphocytes use FOXO1 signaling in a similar keystone role for appropriate differentiation and viral memory responses (Rao et al., 2012; Wang et al., 2014; Zhang et al., 2016). B cells also require active FOXO1 for early development and late activation responses (Szydłowski et al., 2014; Ushmorov & Wirth, 2017), making FOXO1 a key molecule for study in lymphocyte activation.

A standard method for examining FOXO1 localization in lymphocytes is subcellular fractionation and Western blotting (Ishikura et al., 2016; Lin et al., 2017). However, this method requires a large numbers of cells, is dependent on the purity and viability of the starting cell population, and is more qualitative than quantitative. When studying human lymphocytes, many different functional subsets such as effector lymphocytes and regulatory lymphocytes may exist within the population, and can differentially use various signaling molecules. Subcellular fractionation requires at least 3×10^6 cells for an accurate analysis (Barteneva et al., 2012; Goldfine et al., 1977). However, an example of a rarer peripheral blood subset, human Tregs are only a small fraction (~1%) of lymphocytes in the peripheral blood (Golding et al., 2013; Thornton et al., 2010) and often, the yield is no more than ~200,000 Tregs from 50 cm^3 of whole blood (Weingartner et al., 2017). This small number prevents accurate and quantitative analysis of FOXO1 localization by the classic method of subcellular fractionation and Western blotting in human lymphocyte subsets such as Tregs.

To dissect the role of FOXO1 in various lymphocyte subsets, a method is needed that can make use of a small numbers of cells and provide both qualitative and quantitative information. Additionally, when cells are fractionated for WB analysis, there are multiple cell types within a heterogenous population. This can blur the effect of changes in protein signal within a population (Huang, 2009). If an alternative approach could also distinguish different subsets within a parent population, and characterize the sub-cellular localization of native cellular proteins, such as TF, this could offer valuable insights into human lymphocyte developmental stage or activation status (Barteneva et al., 2012).

We evaluated imaging flow cytometry (IFC), which combines the qualitative precision of microscopy with the quantitative measure of flow cytometry, as a reliable method of assessing FOXO1 localization in human lymphocytes. IFC, like classic flow cytometry, can analyze single cells within a population based on specific fluorescence staining, while providing qualitative images of each single cell (Basiji et al., 2007; McGrath et al., 2008). This allows for gating and analysis of the subcellular distributions of any chosen parameter within a specific subpopulation. A crucial consideration is the fact that IFC has previously been described as a reliable method for quantifying nuclear translocation (George et al., 2006), specifically the nuclear translocation of NF-κB, a key lymphocyte transcription factor (Maguire et al., 2011). Another group (Trinité et al., 2014), has previously stained isolated human CD4+ T cells for native FOXO1 and used IFC to assess the effect of HIV infection on FOXO1 localization within memory and naïve CD4+ cells, showing that HIV infected CD4+ cells have decreased nuclear FOXO1 compared to HIV negative cells. While these authors did demonstrate that IFC could be used to visualize native FOXO1 localization in two different, gated populations of human CD4+ T cells, to date, there is limited information regarding using IFC to assess dynamic changes in FOXO1 localization in either total human CD4+ T cells, or in T cell subpopulations cultured within a population of total human peripheral blood mononuclear cells (PBMCs).

In this report, we use a human CD4+ lymphocyte line, HuT102, which we demonstrate has baseline nuclear FOXO1, and dynamically alter FOXO1 localization using a T cell receptor (TCR) signaling mimic with or without Akt inhibition. We assessed FOXO1 localization via the standard method of subcellular fractionation and WB as well as our protocol for imaging flow cytometry. We also examined IFC as a method for reliably detecting qualitative and quantitative changes in native FOXO1 localization in primary human CD4+ and CD8+ T cells over time, when total PBMCs were cultured with a TCR signaling mimic. Thus, we report the validation of IFC as a reliable method for quantitative analysis of dynamic native FOXO1 localization in human lymphocytes on a per cell basis within user defined subpopulations. Advantages of this method include the small number of cells needed, the ability to gate on subpopulations, and the proven capability to detect differences in nuclear translocation of human TFs.

2. Materials and methods

2.1. HuT102 cell culture

HuT102 cells (kind gift of Dr. Arnob Banerjee, UMSOM, authenticated by UMSOM Biopolymer Genomics Core Laboratory - 100% similarity to ATCC reference strain TIB-162) were maintained in complete RPMI 1640 medium at 37 °C at a concentration of between 2×10^5 and 1.5×10^6 cells per mL.

2.2. In vitro HuT102 stimulation

HuT102 cells were cultured at 37 °C at a concentration of 1×10^6 cells per mL with either phorbol 12-myristate 13-acetate (PMA)/ionomycin (PMA/I) (eBioscience™ Cell Stimulation Cocktail (500×) #00-4970-03) or an Akt inhibitor (Akt inhibitor VIII, Isozyme-Selective, Akti-1/2 - CAS 612847-09-3 – Calbiochem, #124018) for a time course of 0.5 to 24 h.

2.3. Subcellular fractionation and Western blot analysis

Subcellular fractionation of HuT102 cells was performed according to manufacturer's instructions using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). FOXO1 localization in the nuclear/cytoplasmic extracts was confirmed via standard Western blot technique. Antibodies used include anti-nuclear lamin B (Cell Signaling Technology; (D9V6H) Rabbit mAb #13435), anti-FOXO1 (CST; (C29H4) Rabbit mAb #2880), and anti-β-actin (Pierce Beta-actin loading control monoclonal antibody #MA5-15739). Image analysis was done in ImageJ (Schneider et al., 2012) and nuclear to cytoplasmic ratio of FOXO1 was calculated via the following equation: Net nuclear FOXO1/Net cytoplasmic FOXO1.

2.4. Primary human T cell culture and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated over a Ficoll gradient from human buffy coats (kind gift of Dr. Scott Strome, UMSOM) and were cultured in complete RPMI 1640 medium at 37 °C at a concentration of 1 × 10 (George et al., 2006) cells with either media alone or PMA/I (eBioscience™ Cell Stimulation Cocktail (500×) #00-4970-03) for a time course of 0.5 to 24 h.