Characterization by mass cytometry of different methods for the preparation of muscle mononuclear cells

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Biological processes that are mediated by cell–cell interactions in heterogeneous populations are best approached by methods that have single cell resolution. Most of these methods rely on the preparation, from solid tissues, of cell suspensions by enzymatic digestion, followed by analysis of single cell reactivity to an antibody panel that allows the discrimination of cell populations and characterization of their activation state. Thus for any specific biological problem, both efficient and at the same time mild, protocols for cell separation, together with tissue specific panels of antibodies, need to be developed and optimized. Here we characterize an antibody panel that permits the discrimination of mononuclear muscle cell populations by mass cytometry and use it to characterize the cell populations obtained by three different cell extraction procedures from muscle fibers. We show that our panel of antibodies, albeit limited and incomplete, is sufficient to discriminate most of the mononuclear muscle cell populations and that each cell extraction method yields heterogeneous cell populations with a different relative abundance of the distinct cell types.

Introduction

Multiparametric single cell analysis is the method of choice for studying biological phenomena in heterogeneous cell samples. Traditional single cell approaches include fluorescence microscopy and flow cytometry. These technologies, however, are limited by the number of available fluorophores and by the overlap in their emission spectra. Thus, only a limited number of readouts can be measured simultaneously.

To overcome this problem, mass cytometry, a novel single cell technology, has been recently developed. Mass cytometry is a highly multi-parametric technology that enables probing of single cell events, by labelling cell surfaces and intracellular antigens with up to 40 antibodies tagged with stable heavy metal isotopes [1]. This technology exploits the possibility to label cells with antibodies as in flow cytometry, but it adds the spectral resolution of Time-of-Flight (TOF) mass spectrometry. Isotopes of the same element differing by a mass unit can be reliably distinguished [2]. In fact, the sharp mass peaks obtained by TOF inductively coupled plasma mass spectrometry eliminate the problems of spectral overlap typical of fluorescence based flow cytometry. Based on these characteristics, mass cytometry enables the detection and characterization of rare and heterogeneous cell populations, by measuring a large number of parameters at the single cell level. This type of analysis is relatively effortless when applied to liquid tissues, but since our interest was on skeletal muscle, we had to develop a specific protocol for mass cytometry analysis of a compact, solid tissue.

Adult skeletal muscle is a relatively complex tissue, which has the ability to self-renew and to self-repair in response to mechanical or chemical damage, stress caused by genetic mutations or increased workload. The regenerative process is orchestrated by different populations of resident mononuclear cells, which directly or indirectly contribute to maintain myofiber homeostasis. The process of myofiber regeneration can be studied *ex vivo* by

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co-cultivating mononuclear cells after releasing them by enzymatic digestion from the extracellular matrix surrounding the muscle fibers.

The preparation of single cell suspensions from complex tissues, such as muscle, requires the application of proteolytic digestions, in order to free cells from the connective compartment. The enzymes commonly used have a proteolytic activity directed against the collagen and the proteoglycans, components of the connective extracellular matrix. Different protocols, relying on different enzymatic activities, for digestion of the extracellular matrix yield different distributions of mononuclear cells. An ideal extraction method should efficiently free cells from muscle fibers and extracellular matrix while limiting the modification of their physiology and of the structures of the proteins that decorate their surface. The characterization of the cell populations that are yielded by different extraction methods is of fundamental importance in standardization and optimization of experiments. The different cell populations in the muscle are defined by the combinatorial expression of CD markers on the cell surface. The identification and the abundance of the muscle populations depend on the combinations of antibodies used [3,4].

The main players in the process of muscle regeneration are satellite cells, a progenitor cell population that represents 2–5% of the sublaminar nuclei [5]. It is commonly accepted that the paired box transcription factor Pax7 is a specific marker for satellite cells [6,7]. However, they can also be identified by a specific reactivity pattern when challenged with other antibodies. For instance, they are stained by antibodies against cell surface receptor α-7 integrin, while they do not react with antibodies against leukocyte common antigen CD45, endothelial marker CD31 and stem cell antigen 1, Sca1 [5].

In addition to satellite cells, a variety of skeletal muscle progenitor cells have been identified residing in the muscle interstitium or recruited from other compartments following injury. These cells are either endowed with myogenic potential or have accessory function during muscle regeneration. Other mononuclear cells include interstitial cell populations, that is, myogenic and endothelial cell progenitors identified in the interstitial space of murine skeletal muscle [8], vessel-associated stem cells [9] and bone marrow-derived stem cells [10], among others.

Fibro-adipogenic progenitors (FAPs) are an important class of muscle interstitial cell, with a fibrotic and adipogenic potential, which play a positive role in muscle regeneration, while also contributing to fibro-adipogenic degeneration of skeletal muscles [11]. This heterogeneous population is characterized by the expression of Sca1, a common interstitial marker; they are also negative for CD31, α-7 integrin and CD45. Recently, a new population characterized by the same expression markers as FAPs and located in the interstitium, has been described. This population expresses the cell stress mediator PW1 and is negative for other markers of muscle stem cells such as Pax7. For these reasons the population was named PICs, for 'PW1+/Pax7− interstitial cells'. However, differently from FAPs, PICs have a myogenic potential *in vitro* and contribute directly to skeletal muscle regeneration [12]. Vessel-associated stem cells are heterogeneous multipotent progenitors of mesodermal origin, which display a diverse differentiation potential, thus contributing in different ways to muscle regeneration. They include mesoangioblasts [13], pericytes [14] and myo-endothelial cells [15], which are all characterized by a specific set of surface antigen markers. Finally, immune system cells play a pivotal role in muscle regeneration, since polymorphonuclear leukocytes and monocytes are responsible for the initial phases of the process and for the activation of committed progenitors [16–18].

The aim of this work was to assemble and characterize an antibody panel for the discrimination of muscle progenitor-cell populations by mass cytometry. This panel is used here to assess different mononuclear cell extraction protocols for studying the complexity of skeletal muscle tissue. In particular, we have designed a panel of eight antibodies, directed against antigens of relevance in myology. We show that this collection of antibodies, albeit limited and incomplete, is suitable for the identification of most of the main skeletal muscle populations by mass cytometry. Moreover, by comparing three different enzymatic extraction methods, we conclude that, according to the protocol utilized, the yields of the distinct cell types and their heterogeneity vary significantly.

Materials and methods

Antibody panel

From a literature search, a list of antigens was compiled that decorate the membrane of muscle mononuclear cell populations. We tested a large number of commercial antibodies. Those that showed sufficient reactivity and specificity after coupling to heavy metal isotopes are listed in Table 1. Other antibodies employed are listed in Table 2.

TABLE 1

List of metal tagged antibodies used in the mass cytometry experiments.

Antibody	Metal	Clone	Fluidigm Cat#	Population
Rat anti-mouse CD45	175 Lu	30-F11	3175010B	Leukocytes (immune system cells)
Rat anti-mouse CD31	165 Ho	390	3165013B	Endothelial and vessel-associated cells
Rat anti-mouse CD11b	172 Yb	M1/70	3172012B	Granulocytes, monocytes and macrophages
Rat anti-mouse CD117 (c-kit)	166 Er	2B8	3166004B	Hematopoietic Stem Cells
Rat anti-mouse F4/80	146 Nd	BM8	3146008B	Macrophages (mature)
Rat anti-mouse Ly-6A/E (Sca-1)	164 Dy	D7	3169015B	FAPs, PICs and endothelial cells
Rat anti-mouse TER-119	154 Sm	TER-119	3154005B	Erythroid cells
Mouse anti-PE	156 Gd	PE001	3156005B	*

* The PE001 secondary antibody clone reacts with phycoerythrin (PE), which is conjugated to anti-mouse integrin α-7 primary antibody (Table 2) for the identification of satellite cells.

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