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Single Cell Multiplex Protein Measurements through Rare Earth Element Immunolabeling, Laser Capture Microdissection and Inductively Coupled Mass Spectrometry

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Abstract

Complex diseases such as heart disease, stroke, cancer, and aging are the primary causes of death in the US. These diseases cause heterogeneous conditions among cells, conditions that cannot be measured in tissue homogenates and require single cell approaches. Understanding protein levels within tissues is currently assayed using various molecular biology techniques (e.g., Western blots) that rely on milligram to gram quantities of tissue homogenates or immunofluorescent (IF) techniques that are limited by spectral overlap. Tissue homogenate studies lack references to tissue structure and mask signals from individual or rare cellular events. Novel techniques are required to bring protein measurement sensitivity to the single cell level and offer spatiotemporal resolution and scalability. We are developing a novel approach to protein quantification by exploiting the inherently low concentration of rare earth elements (REE) in biological systems. By coupling REE-antibody immunolabeling of cells with laser capture microdissection (LCM) and ICP-QQQ, we are achieving multiplexed protein measurement in histological sections of single cells. This approach will add to evolving single cell techniques and our ability to understand cellular heterogeneity in complex biological systems and diseases.

Keywords: Laser capture microdissection; Inductively coupled mass spectrometry; Rare earth elements

Introduction

Our interest in developing an approach for single cell protein measurement grew out of our ongoing studies of focal mitochondrial defects in aging skeletal muscle [1]. Aging is the primary risk factor for the primary causes of death in the United States [2] and like many complex biological processes and diseases such as cancer, heart disease, and neurodegeneration, aging occurs heterogeneously at the cellular and tissue level [3-6]. The focal nature of these processes is reflected in a variety of histopathological changes such as fatty infiltration, fiber atrophy, inflammation and fibrosis. Tissue homogenate studies lack a reference to structural changes and suppress signals from unique, rare events. The heterogeneous distribution of damage necessitates the use of single cell approaches that provide increased sensitivity, spatial resolution, multiplexing and scalability.

Traditional approaches to protein measurement include Western blots, enzyme-linked immunosorbent assay (ELISA), mass spectrometry and immunofluorescence [7]. Approaches such as Western blots, mass spectrometry and ELISA require protein fractions from tissue homogenates that typically require gram to milligram tissue quantities. The homogenization procedures destroy any spatial resolution inherent to the original tissue samples and the tissue amounts required result in the sampling of millions of individual cells. Thus, protein signals from cells in a unique local environment or cells undergoing a rare phenomenon (e.g., malignant transformation, apoptosis, senescence) are lost in the background. Immunofluorescent approaches, such as confocal microscopy provide spatial resolution, but the measurement of multiple proteins within a pathway is limited by the spectral overlap of the fluorophores [8].

A recent approach used REE not normally found in biological systems as antibody tags in atomic mass spectrometric analysis of single hematopoetic cells – termed single cell "mass cytometry" [9]. Using this approach, 34 parameters were analyzed simultaneously in single cells. The use of heavy-metal immunolabeling has a number of distinct advantages to immunohistochemistry (IHC) and IF approaches including opportunities for absolute quantitation, lack of signal overlap, complete lack of "auto-fluorescence" or background and adaptability to high-throughput analyses.

We have adapted the mass cytometry approach for use in histological tissue sections by using laser capture microdissection (LCM) to isolate defined numbers of REE-antibody-labeled cells. This approach allows analysis of single or multiple targeted cells from tissue samples of solid organs and archived pathological specimens. Coupled with single cell DNA and RNA methods, this approach will add to elucidating cell-to-cell variation and rare biochemical conditions, which may have important consequences in complex biological pathways and diseases.

Materials and Methods

Animals and tissue collection

Male C57BL/6 mice were purchased from the National Institute on Aging colony at six months of age. We chose male mice to allow us to compare our LCM-ICP-QQQ measurements to our previous single

cell studies in male mouse tissues. Mice were given free access to water and food and euthanized by carbon dioxide inhalation with death confirmed by usual methods. The quadriceps femoris muscles were dissected from each animal, embedded in OCT mounting media (Miles Inc.) and frozen in liquid nitrogen. Samples were stored at -80°C until sectioned. All animal experiments were approved by the UCLA Institutional Animal Care and Use Committee.

Tissue staining with REE

Mouse quadriceps femoris muscle samples were raised to sectioning temperature of -16°C. Ten micron thick sections were obtained and placed on Arcturus PEN membrane glass microscope slides (Life Technologies). Sections were stored at -80°C until immunolabeling was performed. REE labeled antibodies were prepared and purified according to the manufacturer's instructions (DVS Sciences). Anti-mouse fast myosin antibody (MY-32, Sigma) was tagged with 141Pr. Anti-mouse GAPDH antibody (G9545, Sigma) was tagged with 171Yb. Anti-mouse interleukin-6 (IL-6) antibody was purchased as already tagged with 167Er from the manufacturer (DVS Sciences). MAXPAR Nucleic Acid Intercalator (DVS Sciences) labeled with 103Rh was used to stain cell nuclei. Immunostaining was performed as described previously [1]. Stained slides were dehydrated quickly through an ethanol and xylene series and dried completely before LCM.

Laser capture microdissection of individual muscle fibers

Skeletal muscle fibers were captured using a Leica LMD7000 laser dissecting microscope. Dissections were done using the 20x objective with laser settings of power=25, aperture=17, speed=10, balance=15, head current=100 and pulse frequency=800. Single fibers were collected (Figure 1). Fibers were captured into individual wells of a 48-well microslide. Each captured sample was visually inspected to confirm capture. Captured cells were digested with one microliter of 70% nitric acid and digested samples taken up in nine microliters of water. Samples were stored at 4°C until analysis by ICP-QQQ.

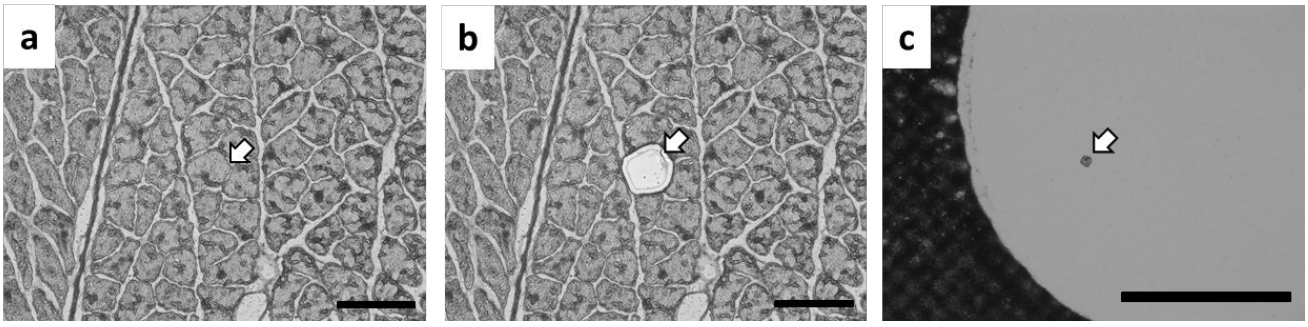

Figure 1: Laser capture microdissection of an individual skeletal muscle fiber before capture (a), the tissue section after capture (b) and the captured fiber (c) Arrow denotes the targeted fiber (a), the selective laser cutting of only the targeted fiber (b) and the captured fiber (c). Black bar is 50 microns (a and b) and 500 microns (c).

ICP-QQQ of REE-immunolabeled muscle fibers

To the Agilent 8800 ICP-QQQ, ten microliter samples were either injected via syringe pump or by an Agilent 1260 Cap-LC at a flow rate of 10 microliter/min. The ICP-QQQ was equipped with a low-flow nebulizer and a total consumption spray-chamber. Sensitivity and ion lens voltages were auto-tuned via MassHunter 4.1 software using 1 ppb tune solution (Agilent, DE). Typical sample introduction parameters were used; 1550W RF power, 1 L/min carrier gas, 0.15 L/min dilution gas, 8 mm sampling depth, and analysis was performed in TRA mode with 0.2 sec integration time. To ensure the entire sample was delivered to the ICP-QQQ, the injected samples were bracketed by air injection.

Statistical analysis

All data with normal distribution were presented as means ± SEM calculated with GraphPad Prism software.

Results

ICP-QQQ of REE and protein measurement

Because of our interest in age-related changes to single muscle fibers, we applied the LCM-ICP-QQQ technique to histological sections of mouse skeletal muscle. We immunolabeled muscle sections with the three REE-labeled antibodies (i.e., antibodies against mouse myosin, IL-6 and GAPDH) and an REE-labeled DNA intercalator. We used laser microdissection to capture five randomly selected muscle fibers from the immunolabeled section and a representative fiber capture is shown in Figure 1. Each captured fiber was digested and analyzed as described in Materials and Methods. As demonstrated in Figure 1, the LCM approach facilitates the capture of a single cell without contamination by surrounding cells and the ability to confirm cell capture before further analysis on the ICP-QQQ.

The Agilent 8800 ICP-QQQ measured the REE signal from each labeled antibody and the DNA intercalator in individual muscle fibers captured by LCM. Table 1 shows the ICP-QQQ data from five representative single muscle fibers. Highest counts were detected for 141Pr and 103Rh that correspond to single cell myosin and DNA content, respectively, while lower counts were detected for 167Er and 171Yb that correspond to single cell IL-6 and GAPDH content. The presence of REE in biological samples is extremely low, resulting in negligible ICP-QQQ background from REE. The average signal to noise ratio across the five samples and the four REE tags in Table 1 was 85. Negative controls included muscle fibers laser captured from mouse muscle sections that were not immunolabeled with REEs and these showed no detectable signals (data not shown).

Discussion

We demonstrate a novel approach for multiplex protein measurements in single cells isolated from histological sections. Our initial data, shown in Table 1, demonstrate the ability to detect REE tagged antibodies for three different proteins and an REE tagged DNA intercalator in single mouse muscle cells. The higher counts for the 141Pr and 103Rh correspond to the expected higher content of myosin, a primary component of the sarcomere, and DNA in the nuclei of these multinucleate cells as compared to the lower counts for 167Er and 171Yb representing IL-6 and GAPDH, respectively, which are known to be present at lower levels in these cells. The variability in 141Pr (myosin) signal is likely due to fiber size differences as is evident in Figure 1, while the variability in the 103Rh (DNA) signal is likely due to differences in nuclei number between different cells. We are developing protocols to normalize the REE counts to cell size or other housekeeping proteins as is routinely done with protein detection in Western blots.

REE label	Fiber 1	Fiber 2	Fiber 3	Fiber 4	Fiber 5	Average counts[b]
^{141}Pr-Ab-Myosin	2562[a]	2726	2835	3198	1976	2659 ± 200
^{167}Er-Ab-IL6	178	273	345	299	233	266 ± 28
^{171}Yb-Ab-GAPDH	105	126	160	138	110	127 ± 10
^{103}Rh-DNA	877	712	1482	776	1132	996 ± 141

[a]Data are represented as counts for each respective mass analyzed (103, 141, 167, and 171 AMU).

[b]Average counts for each REE label ± SEM.

Table 1: ICP-QQQ data from single, REE-labeled muscle fibers.

The recently published approach of single cell "mass cytometry" is currently limited to the study of cells in suspension [9]. LCM allows application of this approach to a wider range of tissue samples and standard tissue sections. These sections can be from any type of pathological samples including biopsies and fine-needle aspirations. The use of LCM also provides access to archived formalin-fixed, paraffin-embedded tissues. We used a standard tissue section immunolabeling protocol for the antibodies and DNA intercalator in this study and this protocol may need to be optimized for each antibody as would be done for standard immunolabeling. Because the REE tagging does not interfere with standard immunofluorescent or immonhistochemical secondary labeling or detection, antibody validation protocols do not need to be altered. With the availability of 32 unique REE labeling kits and the ability of generating different panels of antibodies, there is extensive capacity for multiplex protein measurement. We demonstrated only three antibody labels and one DNA intercalator, but are expanding this to higher levels of multiplexing.

Challenges with this approach include sample delivery, validation and scalability. We used microliter volumes of nitric acid or other buffers for cell lysis, which are loaded into the ICP-QQQ. Consideration must be given to non-specific binding of digested proteins to the sample introduction system, contamination between samples when loading sequential samples, and introduction of control or validation samples. The addition of blocking solutions and different mobile phases may prevent non-specific binding, which will likely be tissue specific. Similar steps must be taken to avoid cross-sample contamination when loading multiple samples through the same sample delivery system. Validation of the single cell ICP-QQQ results is challenging because most other single cell protein measurement approaches are semi-quantitative or qualitative. Specificity could be tested in cells types with known differential protein expression such as slow and fast twitch muscle fibers and antibodies for different myosin types in these fibers. Transgenic animals with known levels of target protein expression could be used to test sensitivity differences between biological samples [10]. With the study of single cells, scalability is important to facilitate building cell profiles from large numbers of single cells. We increased the LCM throughput with the use of a multi-well slide for capturing 48 individual samples for subsequent digestion and are assessing different methods for high-throughput ICP-QQQ sample delivery.

We are making a number of improvements to the LCM-ICP-QQQ approach. We are expanding our panel of labeled antibodies to allow us to examine more proteins in single cells. Panels are in development to examine entire cellular pathways (e.g., insulin signaling) or multi-component protein complexes (i.e., the dystroglycan complex affected in muscular dystrophies). The laser capture approach facilitates the study of other tissue components in histological sections. In addition to single cells including focal areas of fibrosis, inflammation and fatty infiltration, niches not easily studied by homogenate approaches. LCM of organelles such as mitochondria [11] open the possibilities of subcellular protein measurement by the LCM-ICP-QQQ method.

Our approach to measure single cell protein content using REE immunolabeling, LCM and ICP-QQQ is combining standard and emerging techniques to facilitate studies in a wide variety of biological samples at the single cell level. With other single cell analyses, this approach will help uncover heterogeneous cellular events in complex diseases and add to an integrated view of the underlying pathology.

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