Single Cell MALDI-MS Imaging of Lipids and Proteins in Senescent Fibroblasts

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ABSTRACT: In this study, we evaluate the lipidomic and proteomic profiles of human lung fibroblasts (hLFs) to interrogate changes occurring due to senescence. To study single cell populations, a comparison of cell adhered onto slides utilizing poly-D-lysine versus centrifugal force deposition was first analyzed to determine whether specific alterations were observed between preparations. The poly-D-lysine approach was than utilized to interrogate the lipidome of the cell populations and further evaluate potential applications of the MALDI-IHC platform for single-cell level analyses. Altogether, our results show the ability to detect lipids implicated in senescence and alterations to protein expression between normal and senescent fibroblast populations. This report is the first time that the MALDI-IHC system has been utilized at a single-cell level for analyzing the expression of proteins.

INTRODUCTION

Fibroblasts are a diverse set of mesenchymal cells found primarily in the stroma and compose the framework of organs, glands, or other structural components.[1] The cells demonstrate an elongated shape, which enables the formation of cellular adhesions. Fibroblasts can generate adhesions to either a solid substratum or other cells to develop into their stereotypical branched cytoplasm architecture. When lacking or releasing these adhesions, the cell retracts into a roughly spherical form.[2] One of the most notable markers for fibroblasts is the production of cytoplasmic filament vimentin. Vimentin has been reported to play an integral role in the coordination of fibroblast proliferation, collective cell migration, and in wound repair response.[3] Fibroblasts also are effector cells in fibrotic diseases such as Idiopathic Pulmonary Fibrosis characterized by excessive production and deposition of extracellular matrix.[4, 5]

Aging is a complex process occurring due to changes at the biological, biophysical, and biochemical levels, in part due to the accumulation of senescent cells. Senescence is a cell fate decision, characterized by cell cycle arrest, resistance to apoptosis and the senescence-associate secretory phenotype. Although senescence has benefitial physiological effects during development, wound healing, and in cancer prevention; persistence of senescence contribute to the development of fibrotic and age-related diseases[6]. Several triggers of senescence have been described, including the gradual loss of telomeres due to successive cell division a process known as replicative senescence[7] or the exposure to DNA damaging agents such as bleomycin[8].

Integrating single-cell proteomics and lipidomics can provide insight into mechanisms of senescence and disease. Rapidly developing technology within the field of mass spectrometry imaging (MSI) has advanced the technique to the routine interrogation of single cells[9]. In 2022, Cuypers et al. demonstrated the potential for utilizing single cell lipidomic analyses using MSI to differentiate between epithelial cell types and further utilize the unique lipid signals to differentiate between cell lines within a tumor xenograft model[10]. Similarly, Capolupo and coworkers determined that lipid signals in fibroblasts could be used to determine the transcriptional programming of dermal cells[11]. While lipid analysis is rapidly evolving across a multitude of MSI platforms, proteomic imaging, in particular intact protein imaging, has proven to be more challenging. Herein we discuss the utilization of a matrix-assisted laser desorption/ionization immunohistochemistry (MALDI-IHC) platform[12] for targeted proteomics paired with lipidomic analysis of fibroblasts. We examine fibroblasts with and without induced senescence to determine changes at a single-cell level to gain further insight into cell-to-cell variation.

EXPERIMENTAL:

Preparation and Data Collection of Primary Human Lung Fibroblasts. Primary human lung fibroblasts (hLFs) were isolated from healthy young donor and healthy old donor tissues from the Comprehensive Transplant Center

(CTC) Human Tissue Biorepository at The Ohio State University with Total Transplant Care Protocol informed consent and research authorization form. A table of patient demographics can be found in Figure 1. CTC operates in accordance with NCI and ISBER Best Practices for Repositories. Details on cell culture and treatments, including induction of replicative senescence can be found in the supplementary information.

Fibroblast Preparation for Single Cell MSI. Fibroblasts were prepared for single cell MSI following either previous protocols[10] or modified procedures. Briefly, cultured hLFs were lifted from slides using 0.05% trypsin, and either plated on poly-d-lysine slides for overnight adherence or deposited onto slides using a Cytospin™4 (Thermo Fisher Scientific, Carlsbad, CA) cytocentrifuge. Additional experimental details are included in the supplementary information and Figure 1A.

A

Condition	Age	Sex	Passage
Healthy young donor	25 years old	Male	5
Healthy old donor	63 years old	Male	5
Replicative Senescence	25 years old	Male	13

B

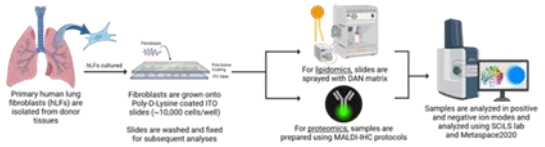

Figure 1: (A) Description of condition, age, sex, and passage number for the hLFs utilized within the studies. (B) Experimental overview for preparation of hLFs for subsequent lipidomic and proteomic analyses. Workflow created using BioRender.com.

Preparation for Protein Analysis Utilizing MALDI IHC Probes. For the analysis of intact proteins, fresh hLF samples grown on poly-D-lysine slides were immediately prepared for MALDI-IHC analysis utilizing the protocol provided with minor adaptations. Details on the procedure can be found in the supplementary information.

Matrix Application for Lipid and Protein Analyses. For lipid analysis, slides were coated with 1,5-Diaminonapthalene (DAN) matrix at a concentration of 10 mg/mL in 90% acetonitrile using an HTX imaging M5 TM-Sprayer (Chapel Hill, NC). For AmberGen protein analysis, slides were coated with α-cyano-4-hydroxycinnamic acid (CHCA) at a concentration of 10 mg/mL in 70% acetonitrile with 0.1% trifluoroacetic acid. CHCA matrix was recrystallized using 1 mL of 5% isopropanol in an oven at 55°C for 1 minute and 30 seconds.

Data Collection. MALDI-MSI spectra were acquired using a timsTOF fleX MALDI-2 mass spectrometer (Bruker Daltonics, Bremen, Germany). External calibration was completed using Agilent ESI-TOF calibration standard. Instrument parameters for each experiment can be found in supplementary table 1. For each sample type, 3 areas of approximately 10,000 pixels were analyzed to represent technical triplicate. Data was analyzed in SCiLS lab 2023b and normalized using root mean square (MALDI-IHC) or matrix clusters (lipids). Data segmentation was completed in SCiLS using weak denoising and bisecting k-means analysis. Putative identifications were determined using the LipidMaps-2017-12-12 database within Metaspace2020[13].

RESULTS AND DISCUSSION:

Comparison of Single Cell Preparation Methods. To determine the best methodology for preparing single-cell samples for MSI analysis, a comparison of utilizing poly-D-lysine as opposed to a cytospin preparation was compared. While the cytospin was able to prepare cells faster, the cell architecture of the characteristic fibroblast branching was not observed. As can be observed in Figure S1, when prepared using the cytospin, cells maintain the structures as when they were lifted. In comparison, cells that are allowed to adhere overnight begin to branch outwards while adhering to the slides. Both methods demonstrated similar lipid signals and masses that distinguished the branching cytoplasm and cell nucleus. While the utilization of both cell preparations for MSI are viable, the utilization of poly-d-lysine cells were chosen due to the branching architecture giving a larger area for analysis and being more recapitulative of the fibroblasts native state within tissue samples.

Analysis of Vimentin Expression in Fibroblasts. It has previously been shown that senescent fibroblasts exhibit higher expression of vimentin than young fibroblasts[14]. To determine the utility of the MALDI-IHC platform to ascertain expression levels of vimentin, cell lines were evaluated representing healthy young and old and replicative senescence fibroblasts. As can be seen in Figure 2, signal from mass tagged vimentin is more highly expressed in the senescent cell lines with higher incidence of signal coming from the branching cytoplasm. Increased expression of vimentin levels was confirmed through the utilization of immunofluorescence and western blot analyses.

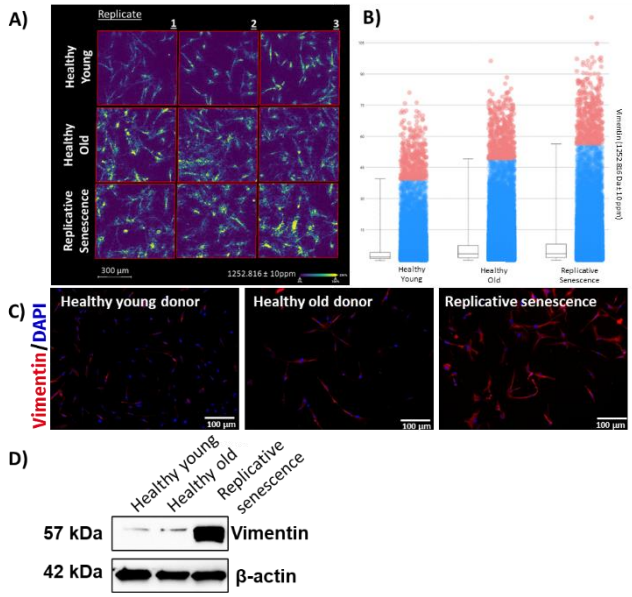

Figure 2. Analyses of Vimentin in hLF. A) Expression of vimentin as observed through the utilization of MALDI-IHC photo-cleavable mass tag at 1252 Da is observed within all datasets with a slight increase in vimentin signal within old and replicative senescence fibroblasts (B). MALDI-IHC confirmed through observation of vimentin expression in IF (C) and WB (D) in hLFs.

Changes in Lipid Signal due to Senescence. Within the observed lipidomic dataset, it was imperative to separate the cell signal from the background to determine changes occurring in cells due to senescence. Normalization techniques were compared (RMS, TIC, and using matrix clusters) and the utilization of a "sprayed standard" of matrix clusters cleaned up the signal for cell segmentation the best. Next, by choosing a small set of ca.20 lipids observed within regions of the cells, unbiased segmentation of data could be obtained for subsequent analyses (Figure S2). For each replicate, data sets were segmented, and average intensities of lipid signals were exported for further analysis within GraphPad using a student's t-test (2-tailed). Very few statistically significant alterations were observed in this mono-culture system, but lipids commonly observed within senescence were detected within the fibroblasts (Figure 3)[15, 16]. Due to the high degree of similarity between these cell lines, it is probable that significant alterations to the lipidome would be difficult to detect through a relative label free quantitation study by MSI. Testing of a larger area of cells in tandem with the utilization of deuterated lipid standards for more stringent quantitation may provide stronger statistical significance between these populations. Furthermore, as lipids levels are controlled through complex mechanisms – it is possible that single cell imaging analyses of monocultures are unable to fully recapitulate significant alterations to lipid levels that are commonly observed within senescent tissues.

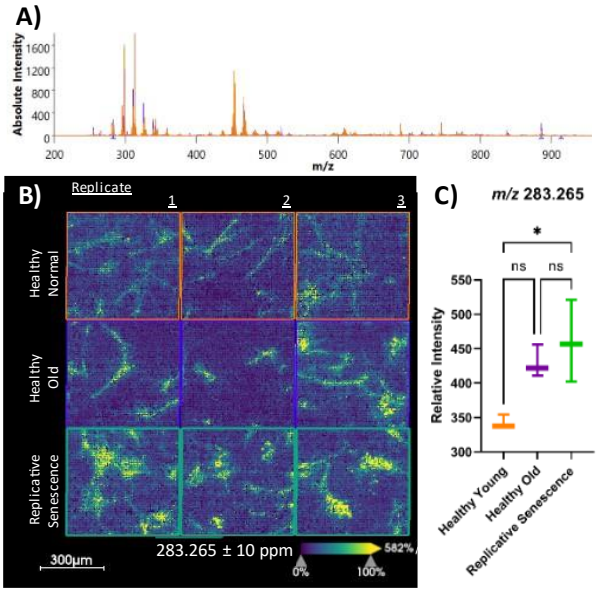

Figure 3. Observation of lipids previously reported within senescence. (A) Comparison of average mass spectra from the segmented cells for each subtype. (Spectra Colors: Orange -healthy young donor, purple - healthy old donor, and green -replicative senescence.) (B) Example of observed signal for putative stearic acid (281.250 Da ± 10 ppm) found to be statistically significant between healthy young donor and replicative senescence fibroblasts (C).

CONCLUSIONS

Advances to mass spectrometry imaging provides a unique opportunity to interrogate omics-based changes at a single cell level. In this study, for the first time, the utilization of MALDI-IHC, confirmed through western blot and IF analyses, showed that the platform can be used to analyze samples at a single cell level with potential for being used as a quantitative marker. MALDI-IHC may also show future potential in detecting protein markers for specific cells within tissue samples to find cellular localizations in complex samples. While we purposefully chose a challenging test set to look for subtle differences in cell lipid composition due to senescence, very few statistically significant lipids were observed within the datasets and further work will aim to more accurately quantitate this molecular subclass. These workflows are robust tools for generating more specific and sensitive signatures of senescence heterogeneity due to cellular populations and will enable more precise mapping of senescence within the lung and other tissues.

ASSOCIATED CONTENT

Supporting Information

Supporting information includes additional experimental details and MSI images, descriptions of segmentation analyses, fibroblast characterization through western blot analyses, and tables detailing the statistical significance determined for interrogated lipid species.

The Supporting Information is available free of charge on the ACS Publications website.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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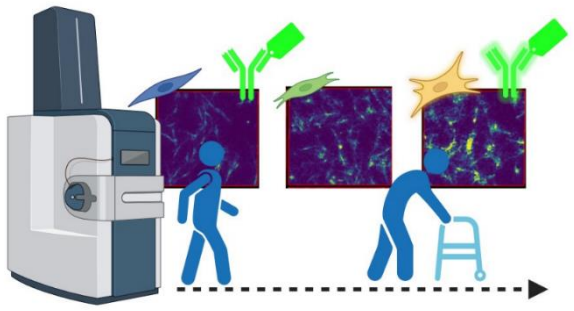
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